Functional group behaviour in reversed phase ion-pair high performance liquid chromatography, using surface active pairing ions.

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FUNCTIONAL GROUP BEHAVIOUR IN REVERSED PHASE ION-PAIR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY, USING SURFACE ACTIVE PAIRING IONS

Submitted by CHRISTOPHER M. RILEY, B. Pharm.

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of the University of Bath

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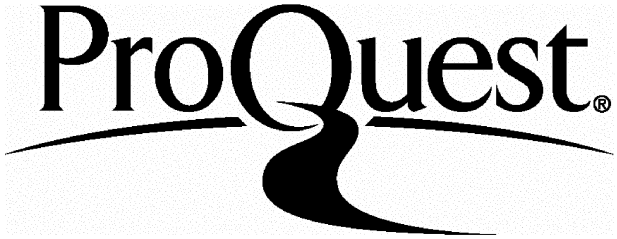

Our experiments are not carried out to decide if we are right, but to gain new knowledge. It is for knowledge sake that we plough and sow. It is not inglorious at all to have erred in theories and hypotheses. Our hypotheses are intended for the present rather than the future. They are indispensable for us in the explanation of the secured facts,to enliven and to mobilise them and above all, to blaze a trail into unknown regions towards new discoveries. (1)

R. Willslatter (1957).

Dedication

To my wife, Pam, without whose strength and encouragement this work would not have been possible.

CONTENTS

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SUMMARY

The behaviour of functional groups having widely different physicochemical character has been examined in reversed phase ion-pair high performance liquid chromatography using surface active pairing-ions of both positive and negative charges. The effects of organic modifier type and concentration, stationary phase carbon loading and chain length, ionic strength, pH, pairing-ion structure and concentration and solute charge on the extrathermodynamic functional group values, have been determined.

Analysis of group behaviour within the framework provided by solvophobic theory is often found to be possible using linear free energy approaches, and it is shown that retention in such systems can be described in terms of ion-pair formation in the mobile phase followed by distribution to the stationary phase. In addition the substituents were found to exhibit linear enthalpy-entropy compensation behaviour, suggesting further that a common retention mechanism can be described for all ionised solutes using these pairing-ions.

These theoretical considerations permitted the optimisation of the separation conditions for the analysis of solutes of pharmaceutical and biomedical interest in environments such as drug formulations and biological fluids. It is shown that chromatographically derived group contribution values may be used to predict bulk phase hydrophobicity properties and may be used directly in drug design models. Conversely, liquid-liquid distribution hydrophobicity parameters can be used for the rationalisation of retention behaviour in reversed phase high performance liquid chromatography using surface active pairing-ions.

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SECTION 1. INTRODUCTION

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1.1 A Brief History of Chromatography

Chromatography has been described as "those processes which allow the resolution of mixtures by effecting separation of some or all of their components in concentration zones on or in phases different from which they were originally present, irrespective of the force or forces causing the substances to move from one phase to another" (2).

Tswett (3,4) discovered 'elution development' chromatography around 1903 when he succeeded in separating a number of plant pigments by elution with petroleum down a calcium carbonate column. However filter paper chromatography ('frontal development') had been described as early as 1861 by Schonbein (5). Tswett's use of the term chromatography appears to date to a treatise on colour (6) in the early 1700s.

Tswett's work went largely unrecognised for two decades (7) until it was rediscovered by Lederer (8) in 1930. There then followed another dormant period of nearly ten years until the full potential of Tswett's early work was realised. During the period from 1940 to 1952 the work of Martin and co-workers (5, 9-13) remains outstanding because their studies not only formed the basis of present chromatographic theory but also predicted the development of chromatography for the next thirty years.

Martin and Synge (9) described the chromatographic process in terms of the height equivalent to one theoretical plate, HETP, a term used previously in fractional distillation. They indicated the future direction of chromatographic research in two, now famous sentences, viz. "the smallest HETP should be obtainable by using very small

particles and high pressure differences across the column", and, "in striving for conditions for uniformity of flow, the high pressures and small particles desirable for smaller HETP have to be abandoned".

These workers were also concerned with the resolution of ionised solutes and indicated the importance of pH control using buffers and the role of complex formation. In the context of this thesis the following statement is especially relevant (5) "we can hope shortly to hear of the modification of partition coefficients by salt formation with added organic acids and bases, to increase their solubilities in non-polar solvents".

With the notable exceptions of thin-layer chromatography and paper chromatography, the full potential of liquid chromatography was not realised until the late 1960s with the advent of high pressure (*sic*) liquid chromatography. Although this later development of column liquid chromatography traditionally has been attributed to the lack of adequate technology in the 1950s and 1960s, Bristow (14) has suggested that this technology was available and the failure to develop column liquid chromatography during this period was due to lack of foresight on the part of instrument manufacturers. Indeed specific examples may be found where the technology of column liquid chromatography though developed was not extended, (e.g. the amino-acid analysers of Moore and Stein (15)).

However with the appearance in the late 1960s of a number of papers reporting the use of pressurised liquid flow and on line detection in column chromatography (16-21), many of the technological problems associated with pumps and detectors were rapidly overcome,

and the first commercial liquid chromatograph was produced by Du Pont Instruments in 1971.

Initially stationary phase materials were of large particle size (more than 30 μm in diameter) and generally pellicular (16-18),and improvements were made with the introduction of porous silica and alumina microparticles of 5 μm and 10 μm diameter (22). Once initial column packing problems had been overcome (23-27) the small HETPs predicted by Martin and Synge (9) were realised.

The introduction of highly efficient columns packed with porous microparticles was only partly responsible for the widespread application of high performance liquid chromatography; of equal importance was the introduction of chemically bonded phases and reversed phase materials in particular (28). Such has been the value of reversed phase high performance liquid chromatography that it is estimated that between 60 to 80% of all applications involve the use of reversed phase materials (29).

1.2 High Performance Liquid Chromatography

1.2.1 Advantages

The advantages of high performance liquid chromatography (HPLC) lie in the combination of sensitivity, reliability, efficiency and speed of analysis found in gas chromatography with the versatility which was to be found in previously developed liquid chromatography. A modern liquid chromatograph resembles closely a gas chromatograph and much of the supplementary technology applied to gas chromatography such as integrators, computers, auto samplers and mass spectrometers have been applied to HPLC.

A major advantage of HPLC over gas chromatography is that, in general, for purposes of resolution derivatisation of polar solutes (29) is not necessary, and the range of detectors available in HPLC means that sensitivities approaching those found in gas chromatography can be achieved. The most widely used detector is the ultra-violet (UV) photometer (30) but others used include electrochemical detectors (31), fluorescence detectors (32), mass spectrometers (33), flame ionisation detectors (34) and ion-selective electrodes (35). Although derivatisation is not required for separation in HPLC, sensitivity can be dramatically enhanced by the formation of strongly UV absorbing (36-38) or fluorescent (39-44) derivatives (either chemical or physicochemical e.g. ion-pairing).

The advantages of reversed phase methods are both chromatographic and practical. In the chemical, biological and pharmaceutical areas, the majority of compounds of interest are relatively large organic molecules having a hydrophobic nucleus with polar functional groups attached. These compounds are prone to peak tailing in adsorption chromatography due to the non-homogenous nature of the stationary phase surface (29); interactions with surface of reversed phase materials are much less specific (29) and homogeneous and the eluted peaks are much more symmetrical. Ease of operation, high chemical selectivity and their compatibility with biological materials and aqueous extracts (45), are other reasons why reversed phase materials are often the stationary phase of choice.

1.2.2 Theory and definitions

Chromatography is a separation technique involving the passage of a mobile phase through a solid or liquid stationary phase or support

which may be filter paper, a thin layer spread on plate or held in a column. For separation to occur the solute components of a mixture must migrate differentially to the stationary phase as they pass through it due to the flow of the mobile phase. As the elution process proceeds the bands broaden due to dispersion and diffusion effects, although solute bands move and separate faster than they broaden. Despite this simple concept by which separations occur, the mechanisms of separation and those factors controlling band broadening are undoubtedly complex and diverse.

a. Band broadening

Theoretically it is assumed in chromatography that the chromatographic column or thin layer is divided into small equally sized plates arranged at right angles to the flow. It was assumed originally (9) that the solute molecules in the mobile phase are in equilibrium with those in the stationary phase. In reality, near equilibrium conditions exist due to the flow of the mobile phase and to account for this a modification of the original theory known as the non-equilibrium theory of chromatography has been developed (46, 47).

When a single solute is introduced into the chromatographic system it broadens and rapidly attains Gaussian dimensions such that the total band width equals four standard deviations of the distribution, 4σ. The number of theoretical plates, N, is given by:

$$N = (t_r.\sigma^{-1})^2 = 16(t_r.w^{-1})^2 \quad (1)$$

$$N = 5.54(t_r.w_{\frac{1}{2}}^{-1})^2 \quad (2)$$

where t_r, w and $w_{\frac{1}{2}}$ are the retention time, width of the base and width at the half height of the peak respectively.

The height equivalent to one theoretical plate (H) is given by:

$$H = L.N^{-1} \quad (3)$$

where L is the length of the column. And the reduced plate height ,h, (48) is given by:

$$h = H.d_p^{-1} \quad (4)$$

where d_p is the diameter of the stationary phase particles.

b. Retention

In thin-layer and paper chromatography, the retention of the solute is measured by the dimensionless R_F value which relates the distance moved by the solute to the distance moved by the mobile phase. The analogous term used to describe retention in gas and column liquid chromatography is the capacity ratio. To avoid confusion with terms used in non-chromatographic areas, the term κ is preferred here to the often used k' term to denote the capacity ratio, and is given by:

$$\kappa = q_s.q_m^{-1} \quad (5)$$

where q_m and q_s are the amounts of solute in the mobile phase and stationary phase, respectively. From this it follows that:

$$\kappa = c_s.V_s(c_m.V_m)^{-1} \quad (6)$$

and

$$\kappa = D.V_s.V_m^{-1} \quad (7)$$

where c and V are the solute concentrations in and volumes of the mobile phase, m, and the stationary phase, s, respectively, and D is the distribution ratio of the solute between the two phases. The capacity ratio is formalised by:

$$\kappa = (t_r - t_o).t_o^{-1} \quad (8)$$

where t_r and t_o are the elution times of retained and unretained peaks respectively.

c. Resolution and Selectivity

For the resolution, R_s, of two solutes (1 and 2) we may write:

$$R_s = 2(t_{r2} - t_{r1}).(w_2 + w_1)^{-1} \quad (9)$$

As t_{r2} tends to t_{r1}, w_1 approximates to w_2, and hence:

$$R_s \simeq (t_{r2} - t_{r1}).w_1^{-1} \quad (10)$$

When R_s equals 1.5 there is less than 1% peak overlap (14). To identify the thermodynamic and kinetic factors responsible for resolution, Eq. 10 may be rearranged to give:

$$R_s = 0.25.\ N^{0.5}.\left[\frac{\alpha-1}{\alpha}\right]\left[\frac{\kappa}{\kappa+1}\right] \quad (11)$$

where the selectivity factor, α, is given by:

$$\alpha = \kappa_2.\kappa_1^{-1} = D_2.D_1^{-1} \quad (12)$$

Equation 11 shows that resolution is dependent mainly on selectivity and that retention and column efficiency are of secondary importance. Equation 12 demonstrates that selectivity depends upon the nature of the mobile and stationary phases but is independent of phase volume ratio ($V_s.V_m^{-1}$), the relevance of which will be discussed in Section 1.2.4(b).

The importance of selectivity prompted Huber (49) to suggest that there is a need to develop a reliable selectivity index, based on structural differences, and hence based on Traube's rule a more valid selectivity term would appear to be τ, where:

$$\tau = \log(\kappa_j.\kappa_i^{-1}) \quad (13)$$

where j and i refer to substituted and unsubstituted molecules respectively. The parameter τ may be regarded as a group contribution term and can be used to describe the effect of structural modification on retention, or to describe steric effects such as optical or geometric isomerism, where j and i would now refer to two isomeric forms. A functional group may contribute positively or negatively to retention, hence τ may be positive or negative unlike log α which is by convention

always positive.

1.2.3 Stationary phases

Liquid chromatography phase systems have been traditionally divided into two classes, namely straight phase and reversed phase, (according to the nature of the mobile and stationary phases). In straight phase the stationary phase is much more polar than the mobile phase, which typically is a mixture of organic solvents. In reversed phase the stationary phase is non-polar and the mobile phase is polar.

In HPLC the stationary phase may be pellicular (16-18, 50), a porous solid such as silica (22, 51, 52), alumina (53) or carbon (54), a resin (55) or a liquid physically bound to an inert support. In addition chemically bonded phases (56, 57) constitute another type and are now the most widely used. After much debate it is considered that bonded phases behave more as solids than as liquids (57, 58).

Bonded phases may be either polymeric, where the stationary phase is polymerised around a solid support, or of the "brush-type" where the bonded moiety is arranged at right angles to the solid surface. Polymeric materials are used rarely because of their poor mass transfer characteristics and their tendency to swell in certain solvents (59-61).

In the "brush type", the bonded moiety is linked to silica by one of four chemical linkages, i.e. Si-O-C, Si-O-Si, Si-C or Si-O-N. Due to the acid and alkali instability of the other linkages the Si-O-Si is the most widely used, and is generally stable in the pH range 2 to 7.5. The instability of the Si-O-Si linkage in alkali limits its usefulness, however Frei and co-workers (62) have shown that such bonded phases are stable

up to three months when organic bases are used. Alternatively inorganic bases such as ammonia may be used if the analytical column is protected by a pre-column (63).

To understand the retention processes and mechanisms in HPLC using bonded phases, it is necessary to appreciate the structures of the bonded phase and the silica support. Silica is a complex polymer matrix of silicon atoms each linked to three oxygen atoms. The fourth valency position of silicon is occupied by a hydroxyl group or a siloxane bridge. To maximise the conversion from the bridge structure to the linked hydroxyl state heating with steam for several hours at 120°C (28) can be used. The silica surface will then contain between 5 and 8 hydroxyl groups per square nanometre of which 3 to 4 will be hydrogen bonded together (14). The remaining silanol groups are potentially available for linkage with a chlorosilane to produce the bonded phase. In practice only 45 to 75% of the free silanols are available, some are completely sterically hindered and some are partially hindered depending on the size of the bonded group (45). When a large moiety such as octadecyl is bonded, a high proportion of completely and partially hindered free silanol groups may remain. The partially hindered free silanols may be removed (or "capped") by reaction with a small chlorosilane such as trimethylchlorosilane.

Both capped and uncapped bonded phases are available commercially. The presence of uncapped silanol groups may be determined by methyl red adsorption (64) or investigating the retention of nitrobenzene using a mobile phase of n-hexane (47). Hammers *et al*. (65) have described a comprehensive method for characterising the silica surface of reversed phase materials however the semi-empirical method of Knox is of more practical use (47) wherein a capacity ratio of nitrobenzene of less

than 0.5 with a mobile phase of n-hexane indicates the absence of any significant silanol groups.

1.2.4 Retention mechanisms

a. Liquid-solid chromatography

In straight phase high performance liquid-solid chromatography (HPLSC) the mobile phase is predominantly an organic solvent mixture and the polar stationary phase is generally silica gel or a modified silica (e.g. amino-silica or cyano-silica), the latter phases having more specific application.

The surface of silica consists primarily of slightly acidic silanol groups which may be associated with water molecules via hydrogen bonding. Silica is heated to 125^{o}C to remove all this associated water and to gain its maximal activity, known amounts of water may then be added (either *in-situ* or prior to column packing) to reduce its activity and eliminate the most active sites which effectively reduces peak tailing. Snyder (66) has shown that the importance of water content decreases as the polarity of the mobile phase increases, and Paanaker *et al.* (67) have demonstrated recently that reproducibility of retention and selectivity is dependent on the water content of the organic mobile phase.

Two mechanisms of retention have been proposed in liquid-solid chromatography using silica stationary phases. Snyder and others (66, 68, 69) developed the solvent competition theory, and Scott and Kucera (70, 71) developed the solvent interaction model. The solvent competition model assumes that the solute and solvent molecules compete for the active silanol sites and the solvent interaction model assumes that the surface of the adsorbent is covered with a layer

of the more polar component of the mobile phase. Above 3% v/v of polar solvent content the surface is completely coated such that non-hydrogen bonding solvents form mono-layers, whereas hydrogen bonding solvents may form bilayers (70, 71). The solvent interaction model assumes that the solutes interact with adsorbed solvent molecules and when the surface is completely coated retention is determined solely by solute-solvent interactions in the mobile phase. Although the solvent competition model is more widely accepted, it is interesting to note that Soczewinski and Golkiewicz (68, 69) have shown that the two models are indistinguishable at higher concentrations of polar solvents.

For reversed phase liquid-solid chromatography (HPLSC) the mobile phase is generally aqueous and ideally the stationary phase has non-polar character. The stationary phase may be an inherently hydrophobic solid such as ceria (72), zirconia (73) or pyrocarbon (54, 74) or an alkyl- (or alkyl-aryl) silica (56). Despite being the most commonly used form of HPLC, the retention mechanism in reversed phase is the least well understood (45). Scott and Kucera (75) have applied the solvent interaction model to show that when using an aquous methanol mobile phase a layer (or bilayer) of methanol is adsorbed onto the non-polar surface. Their model assumes that solutes are retained due to interaction with this adsorbed layer of methanol and helps to explain why increased carbon loading or alkyl chain length leads to increased retention (76-79) due to the increased amount of adsorbed methanol. Scott (28) has shown also that retention is greater for polymeric materials compared with the brush type, presumably due to the greater uptake of methanol within the polymer matrix. Once the surface is completely coated with organic modifier there will be no change in the interaction of the solutes with the stationary phase and retention is

controlled solely by interactions in the mobile phase.

The adsorption of mobile phase components onto the stationary phase results in a decrease in the volume of the mobile phase within the column (V_m). This may introduce errors in the measurement of capacity ratios (80, 81) due to the change in phase volume ratio upon changing organic modifier composition, however no such errors are introduced in the measured τ or α values, which are independent of phase volume ratio (Eq. 13).

Residual silanol groups not only influence the amount of adsorbed mobile phase components (81) but also introduce the possibility for mixed retention mechanisms (28). Since the primary component of the mobile phase is water the residual silica surface will be fully deactivated due to the adsorption of water *via* hydrogen bonding. Scott and Kucera have shown (81) that water is adsorbed as a monolayer onto the residual silanol groups and that methanol is bound as a second, less tightly held, layer.

Several workers (82-84) have suggested that retention and selectivity in reversed phase HPLSC is controlled by mobile phase solubility, and Locke (82) has shown that for closely related solutes the selectivity factor can be given by:

$$\alpha = \gamma_2^{\infty} \cdot \gamma_1^{\infty -1} \qquad (14)$$

where γ^{∞}_1, γ^{∞}_2 are the activity coefficients of solutes 1 and 2 at infinite dilution. This approach assumes that similar solutes have identical adsorption energies and that it is the different solvation energies responsible for the desorption processes that account for the selectivity. Thus, Eq. 14 will not apply to totally unrelated solutes which

can be expected to have different adsorption and desorption energies. Locke's theory is also inapplicable to these unrelated solutes since it neglects the effects of lattice energies on solubility (85).

Alternatively it has been proposed that the interaction of solutes with the stationary phase is due to solvophobic effects of the aqueous mobile phase (86-88). Horváth and co-workers (87, 88) have applied Sinanoglu's Solvophobic Theory (89) to reversed phase high performance liquid-solid chromatography and explained retention as being due to the repulsive forces of the aqueous phase rather than to attractive forces between the solute and the stationary phase.

In the Solvophobic Theory of retention in reversed phase, the solute (S) is retained by reversible complex formation with the hydrocarbonaceous stationary phase ligand (L):

$$S + L \rightleftharpoons SL \qquad (15)$$

Mobile phase — Stationary phase

Therefore three species S, L and SL can exist and may interact with the surrounding solvent. The overall standard free-energy of association (ΔG^{o}_{assoc}) is given by:

$$\Delta G^{o}_{assoc.} = \Delta G_{vdw,assoc} + (\Delta G_{c,SL}+\Delta G_{i,SL}) - (\Delta G_{c,S}+\Delta G_{i,S}) - (\Delta G_{c,L}+\Delta G_{i,L}) - R.T.\ln(RT.(P_{o}.V)^{-1}). \qquad (16)$$

where ΔG_i and ΔG_c refer to the free energy changes associated with the interaction of the species S, L and SL with the solvent and the formation of a cavity in the surrounding solvent. $\Delta G_{vdw,assoc.}$ is the free energy of interaction of the solute and the solvent due to van der Waal's forces in the gas phase. The last term accounts for the entropy change due to the change in free volume and contains the mole volume of the solvent

v) and the atmospheric pressure (P_o). R and T are the gas constant and the absolute temperature, respectively.

By analysis of the various terms in the above equation the following relationship can be derived which describes all the factors affecting retention:

$$\ln k = \frac{\Delta G^{o}_{vdw} + N\Delta A_{\gamma} + 4.84\ N^{0.33}(k^{e}-1)V^{0.66}\gamma}{R.T.} + \ln V_s.V_m^{-1} + \text{constant} \qquad (17)$$

Where ΔA is the area of the solute in contact with the stationary phase, k^e is the ratio of the energy required to form a cavity for a solute molecule in the solvent to the energy required to extend the planar surface area of the solvent by an amount equal to the surface area of the added solute. γ is the surface tension of the mobile phase and N is Avogradro's number. Consequently, retention depends upon the carbonaceous surface areas of the solute and the stationary phase (V_s), absolute temperature, surface tension of the mobile phase and a component equal to the free energy of association of the solute and the solvent due to van der Waal's forces. (ΔG_{vdw}).

Lochmuller and Wilder (76) have suggested that Horváth group's application of Solvophobic Theory is incomplete since it does not describe adequately the contribution of the stationary phase to retention and it does not explain the observation that the alkyl chain length of the stationary phase affects selectivity (58, 77).

b. Liquid-liquid Chromatography

Retention in liquid-liquid chromatography is due to the distribution, or partitioning (*sic*) of a solute into a physically bound liquid stationary phase which is immiscible with the stationary phase. Huber and co-workers have suggested that (90, 91) retention in such

systems may be determined from bulk phase partition coefficients according to Eq. 18 , (see also Section 1.5.3), i.e.

$$\kappa = K_{D.}.V_s.V_m^{-1} \qquad (18)$$

where K_D is the bulk phase liquid-liquid distribution (or partition) coefficient.

For the purposes of this thesis partition is defined as the distribution of a solute between two immiscible liquids such that the interface does not significantly influence the liquid-liquid distribution coefficient. In liquid-liquid HPLC the stationary liquid exists as a thin film and cannot be considered to be a true bulk liquid phase and thus the interphase will significantly affect the retention process. Hence the term distribution is preferred to partition in such systems.

c. Ion-exchange Chromatography

Stationary ion-exchangers may be either polymer resins, such as sulphonated polystyrene (92), or ionisable moieties chemically bonded to silica (56). Due to their swelling capabilities in aqueous solvents polymer resins have poor mass transfer characteristics and give low column efficiencies (92). Ion-exchangers are classified as weak or strong depending on the ionisation constants of the functional groups. Strong ion-exchangers are ionised at virtually all pHs and include quaternary ammonium materials and sulphonic acids. Weak ion-exchangers are ionised at certain pHs and include carboxylic acids and amines.

Retention by ion-exchange is due to competition between the solute ions and mobile phase ions for the oppositely charged stationary ligands. Consequently retention will depend on the mobile phase pH, the concentration of eluent ions (ionic strength) and the relative affinities of

the two species for the stationary phase ligands. The following equilibrium can be envisaged as existing between an eluent and solute ions, A and B, i.e.:

$$A^+_L + B^+_M \rightleftharpoons A^+_M + B^+_L \qquad (19)$$

where the subscripts L and M refer to the stationary phase ligands and the mobile phase respectively. The ion-exchange constant (K_E) is given by (92):

$$K_E = \left[B^+_L\right] \cdot \left[A^+_M\right] \cdot \left(\left[B^+_M\right] \cdot \left[A^+_L\right]\right)^{-1} \qquad (20)$$

The ability of the eluent ions to displace the solute ions, depends on their valency and size (14). Small highly charged ions have the highest displacing ability. Conversely, small highly charged ions are retained more than large monovalent ions.

An important side reaction in ion-exchange chromatography is interaction of the solutes with the stationary support, and when the charged moiety is linked to the support via an alkyl group, retention is modified due to the interaction with such hydrophobic groups. Liao and Vogt (93) have made use of this by preparing a stationary phase which has both ion-exchange and purely hydrophobic alkyl groups.

1.3 Ionic Surfactants

Ionic surfactants are of particular interest in Pharmacy not only as therapeutic agents but also as formulatory adjuvants and preservatives. Ionic surfactants are characterised as having a charged group (or groups) attached to a hydrophobic alkyl chain. Surfactants are adsorbed at interfaces such that the interfacial tension is reduced. This property of surfactants is a manifestation of the hydrophobic effect which was understood qualitatively by Traube in 1891 (94). Traube showed that molecules having a polar group, a long hydrocarbon

chain and a reasonable solubility in water tended to migrate to the surface of the solution. At low concentrations where the interactions between the alkyl groups is minimal the ratio of the concentration of the surfactant at the surface to that in the bulk increases three-fold for each methylene group added to the alkyl chain. This effect could not be attributed to interaction of the alkyl chains but to the lack of affinity of the alkyl chains for the water.

According to Thermodynamic Laws all systems tend to the lowest energy state and to disorder. For a process to be spontaneous, the system must lose an amount of free energy (ΔG) which is related to the total heat or enthalpy change (ΔH) and the entropy change (ΔS) which is a measure of the energy change due to the change in order of the system, such that:

$$\Delta G = \Delta H - T\Delta S \qquad (21)$$

where T is the absolute temperature.

Although superficially the spontaneous adsorption of surfactant molecules will have a positive entropy change due to the increased order of the adsorbed molecules, in fact, except at high temperatures (95) the hydrophobic effect is entropically driven (+ve ΔS) due to the loss of the highly structured water molecules around the alkyl chains. Interestingly, Howarth (96) using nuclear magnetic resonance studies found no evidence of water structuring around alkyl chains in aqueous solution, and described the hydrophobic effect in terms of reduced tumbling of the water molecules at the edges of solvent cavities.

Another important property of surfactants is their ability to form

regularly structured micelles (97). Initially, when a surfactant molecule is placed in water, the water molecules form a cage-like structure around the alkyl chains. As the surfactant concentration rises the dispersion force interactions between the water and the alkyl chains increase leading to an increase in free energy and hence a strain on the water structure. Eventually the water expels the hydrophobic chains resulting in the formation of micelles. The concentration at which this occurs is known as the critical micelle concentration, CMC. Any change in molecular structure which would increase the entropy change will result in a greater hydrophobic effect: for example the CMC of surfactants decreases with increasing alkyl chain length (98, 99).

1.4 Ion-Pairs

Ion-pairs are defined as Coulombic association species between ions of opposite electrical charge. The formation of ion-pairs is dependent on such variables as solvent dielectric, and ion constitution and polarisability. Ion-pairs can be conveniently classified according to the nature of the two ions *viz*: inorganic-inorganic, inorganic-organic and organic-organic.

The failure of sodium chloride to behave as a strong electrolyte in liquid ammonia, led Bjerrum (100) to postulate a theory of ion association based on electrostatic considerations. The association of electrical charges leads to a net partial neutrality, although the formed species do retain some polarity (101, 102). Originally it was considered that ion-pairs could only exist in solvents of low dielectric; however the work of Diamond (103) and later of others (104) has shown that ion-pairs can exist in aqueous solution if both species are sufficiently large and have an appreciable hydrophobic integrity, and

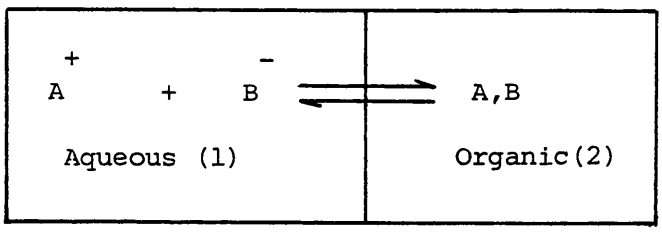
hence ion-pair formation in aqueous solution is due in part to hydrophobic effects.

Within an analytical context, the major property of ion-pairs is their ability to transfer from an aqueous environment to an area of low dielectric and this phenomenon has been exploited extensively in both solvent extraction chemistry (101, 102, 105-107) and in chromatography (108, 109).

1.4.1 Solvent extraction

Many solutes of biological and pharmaceutical interest are weak acids or bases and their extraction from aqueous environments such as biological fluids or drug formulations has proved difficult by conventional extraction procedures. A number of workers, in particular Higuchi *et al.* (101, 102) and Schill and co-workers (105), have applied ion-pairing techniques to improve the extraction of ions into organic solvents.

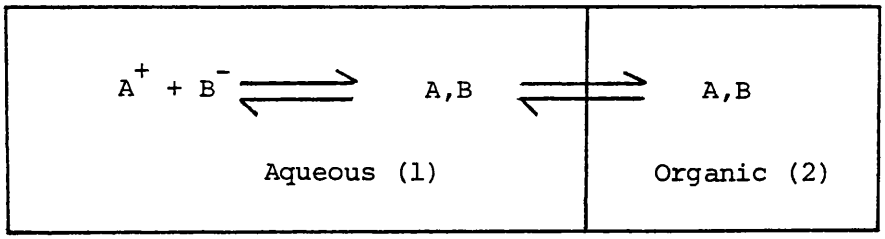
The overall transfer of an ion-pair between two liquid phases will be determined by the extent of ion-pair formation, the nature of the formed ion-pair and the properties of the extracting solvent. Thus Na, Cl will have a much lower solubility in an organic solvent than R_1SO_4,RN, where R and R_1 refer to alkyl groups. The overall transfer of an ion-pair can be represented by Scheme I.



Scheme I

For ion-pairs between large hydrophobic ions, formation can be in the

bulk aqueous phase (95) followed by transfer to the organic phase:



Scheme II

For small ion-pairs, ion-pair formation will be in the interfacial or diffusion layers between the two phases where the solvent dielectric is much lower.

Since ion-pairs are not non-polar, it is more relevant to discuss their extraction in terms of specific solvate formation rather than in relation to solvent dielectric or regular solution theory (101, 102). Higuchi *et al*. (102) found that the extraction of dextromethorphan hydrobromide ion-pairs was greatest into chloroform-cyclohexane mixtures compared with cyclohexanone-cyclohexane or nitrobenzene-cyclo-hexane, despite the former having a much lower dielectric constant. Regular solution theory would tend to predict a greater extraction of the slightly polar ion-pair into the more polar solvent. This is not so because the dextramethorphan hydrobromide ion-pair can be regarded as having a lipophilic cation with low net charge per unit area and a smaller cation with a high net charge per unit area. This species would be solvated in the organic phase and extracted into it by molecules having an exposed positive surface with acidic protons such as chloroform, phenols and alcohols. In addition, strongly hydrogen bonding solvents will have a high extracting ability for ion-pairs.

For the extraction of a number of inorganic anion-organic cation

pairs the distribution ratio increases with the size of the cation (110, 111) and for the extraction of organic anion based ion-pairs the distribution ratio increases by a factor of two for each added methylene in the alkyl chain. The distribution of the ion-pair will also be influenced by side reactions and environmental effects such as temperature, ionic strength, pH etc.. The overall transfer may be given by:

$$nA^{m+}_{aq} + mB^{n-}_{aq} \rightleftharpoons (A_n,B_m)_{org} \qquad (22)$$

where n and m refer to the stoichiometry of the process. The extraction constant ($K_{EX_{A,B}}$) is given by Eq. 23 for this process

$$K_{EX_{A,B}} = \left[A_n,B_m\right]_{org} \cdot \left[A^{m+}\right]^{-n}_{aq} \cdot \left[B^{n-}\right]^{-m}_{aq} \qquad (23)$$

The distribution ratio (D) describes the overall extent of solute transfer and, for monovalent ion-pairs, is given by:

$$D_{A,B} = K_{EX_{A,B}} \left[A^{+}\right]_{aq} \qquad (24)$$

Equations 22 to 24 assume that no side reactions such as ion-pairing in the aqueous phase, secondary equilibria and dimerisation or dissociation of the ion-pair in the organic phase do not influence the phase transfer. The influence of side reactions (105, 109) on the distribution ratio may be accounted for by the use of conditional extraction constants(112, 113).

1.4.2 Thin-layer and paper chromatography

The application of ion-pair extraction principles to thin-layer chromatography (TLC) has been made by numerous workers, in particular Gronningsson and Schill (114, 115).

Since the R_F value of a solute B is given by:

$$R_F = q_{Bm}.(q_{Bm} + q_{Bs})^{-1} \quad (25)$$

where q is the amount of solute, it follows that:

$$R_F^{-1} = 1 + [B]_s.[B]_m^{-1} V_s.V_m^{-1} \quad (26)$$

$$= 1 + D_B.V_s.V_m^{-1} \quad (27)$$

where [B] now refers to concentrations. If the solute is ionised and a pairing-ion A^+ is present in the aqueous phase then the mechanism of distribution can be represented by:

$$B^-_{aq} + A^+_{aq} \rightleftharpoons A,B_{org} \quad (28)$$

For reversed phase systems the pairing-ion is added to the mobile phase and the R_F value is related to the distribution ratio and the bulk phase extraction constant as follows:

$$R_F^{-1} = 1 + D_B^-.V_s.V_m^{-1} \quad (29)$$

$$= 1 + K_{EX_{A,B}}.[A^+]_{aq}.V_s V_m^{-1} \quad (30)$$

For straight phase systems, the pairing-ion is added to the stationary phase and the solute is extracted into the organic mobile phase. In this case the extraction constant is related to the R_F value as follows:

$$R_F(1-R_F)^{-1} = K_{EX_{A,B}}.[A^+]_{aq}.V_m.V_s^{-1} \quad (31)$$

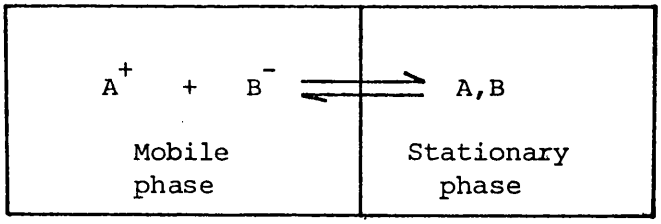
Schill and Gronningsson (115) obtained good correlations between the extraction constants obtained by ion-pair TLC and by bulk phase extractions for papaverine, sercogan and strychnine paired with various inorganic ions. Conversely they showed that optimum separation conditions could be achieved by prior knowledge of the extraction constants and the phase volume ratio (114).

Lepri *et al* . (116) developed the use of surface active pairing ions in thin-layer chromatography, and suggested an ion-exchange mechanism of retention between the solutes and the adsorbed pairing-ions, having previously separated catecholamines (117), food dyes (118) and sulphonamides (119) using conventional thin-layer ion-exchangers. They termed this technique "soap thin-layer chromatography", after Knox & Laird (120) who had previously used the term "soap chromatography" to describe the use of surfactant pairing-ions in reversed phase HPLC (see Section 1.4.3).

In a series of publications Lederer and co-workers (121-127) have described the effects of ion-pairing in paper chromatography (121) and paper electrophoresis (122-127) of metal complexes, inorganic anions and quaternary ammonium neuromuscular blockers. Farulla *et al*. (121) investigated the effect of added ionic surfactants (sodium dodecylsulphate and cetrimide) on the retention behaviour of a series of dyes, and the mechanism of retention was rationalised in terms of an ion-exchange process between the solute molecules and the adsorbed surfactants.

1.4.3 Column liquid chromatography

Generally in column liquid chromatography, ion-pairs will be formed between inorganic-organic and organic-organic ions, and the formation of each type will depend upon the nature of the ions and their immediate environment. For reversed phase techniques the pairing-ion is added to the aqueous mobile phase, and the solute will distribute to the stationary phase as an ion-pair according to Scheme III, i.e.



Scheme III

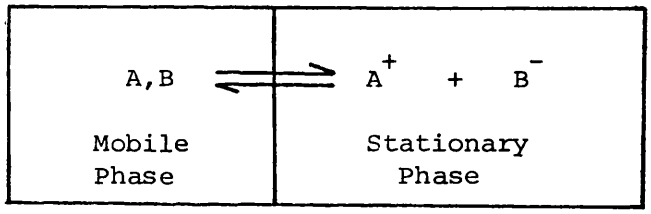
If B^- is the solute ion and A^+ is the pairing ion, then

$$\kappa_B = D_B.V_s.V_m^{-1} \tag{32}$$

and hence from Eq. 30, it follows that:

$$\kappa_B = K_{EX_{A,B}} \left[A^+\right]_{aq}. V_s.V_m^{-1} \tag{33}$$

In straight phase chromatography, the pairing ion is coated as an aqueous layer onto a stationary support and the ion-pair is extracted into the organic mobile phase, i.e.



Scheme IV

In this case the distribution ratio is given by

$$D_B = \left[B^-\right]_{aq} \left[A,B\right]^{-1}_{org} \tag{34}$$

which is reciprocally related to the extraction constant by

$$D_B = (K_{EX_{A,B}} \left[A^+\right]_{aq})^{-1} \tag{35}$$

Hence the capacity ratio is given by:

$$\kappa_B = V_s(V_m.K_{EX_{A,B}} . \left[A^+\right]_{aq})^{-1} \tag{36}$$

Equations 33 and 36 show that the desired capacity ratio may be achieved by the correct choice of the pairing-ion and its concentration in the aqueous phase. Assuming that the phase volume ratio ($V_s.V_m^{-1}$) is unity, for straight phase systems extraction constants in the range 0.2 to 100 are required for good chromatography, whereas

higher values, in the range 10 to 5000, are required for reversed phase systems. Often the phase volume ratio is less than one especially when bonded reversed phases are employed in which case the required extraction constants need to be modified towards higher values.

The derivation of Eqs. 33 and 36 assumes that the concentration of the pairing-ion is in excess of the concentration of the solute ion. Eksborg and Schill (128) have shown that the column load capacity of ion-pair HPLC systems depends upon the extraction constant and the concentration of the pairing-ion. In straight phase systems a low load capacity exists at low pairing-ion concentrations and high extraction constants (128).

Equations 33 to 36 show that the distribution ratio (and hence retention) is dependent on the pairing-ion concentration, the extraction constant and the phase volume ratio; the interrelationships between these parameters are discussed below with regard to the nature of the pairing-ion, the properties of the mobile and stationary phases and various environmental factors. The pairing-ions have been grouped into three classes, a. small hydrophobic and b. inorganic and c. surface active. A comprehensive listing of the types of pairing-ions and solutes examined by ion-pair chromatography and reported in the literature is given in the appendix section.

a. Small hydrophobic pairing-ions

Small hydrophobic ions have been employed in both straight and reversed phase systems. In the former the pairing-ion is coated onto a stationary support such as silica (129) or cellulose (130) (either prior to column packing or *in-situ* (131) as an aqueous buffered solution).

(i) small tetraalkylammonium ions:

The use of small hydrophobic tetraalkylammonium ions stems from the work of Wittmer *et al* . (132) and Schill and co-workers (108, 109). The hydrophobic character of these pairing-ions is altered by changing the length of the alkyl chains and thus a manipulation of the extraction constant, and hence the capacity ratio, is possible.

One problem in the use of tetraalkylammonium ions is the need for buffer salts to control the pH of the system with the possible concomitant problem of competing secondary equilibria. Alternatively the buffer ions may compete with the solutes ions resulting in a salting-in effect (133).

Alkylammonium based ion-pairs having intermediate extraction constants (i.e. 50 to 500) may be chromatographed in either straight or reversed phase systems. Fransson *et al* . (129) have made a comparison of straight and reversed phase separations of some organic acids using tetraalkylammonium ions. They concluded that while both methods gave good possibilities in terms of separation, retention control in the reversed phase mode was easier by changing the type and concentration of the pairing-ion and also by changing the organic modifier in the mobile phase. Wahlund (134) has demonstrated that this latter advantage may be utilised in gradient elution.

Aliphatic alcohols generally constitute a major part of the phase system when using tetraalkylammonium pairing-ions and a problem associated with their use is possible dissociation of the ion-pair in this organic phase, such that:

$$A,B_{org} \rightleftharpoons A^{+}_{org} + B^{-}_{org} \qquad (37)$$

where the dissociation constant ($K_{diss(A,B)}$) is given by:

$$K_{diss(A,B)} = [A^+]_{org} \cdot [B^-]_{org} \cdot [A,B]^{-1}_{org} \qquad (38)$$

Values for the dissociation constant of alkylammonium ion-pairs of around 10^{-3} mol. dm^{-3} have been reported (135) in pentan-1-ol. If A,B is the only ion-pair in the organic phase, then the pairing-ion concentration in the organic phase will vary with solute concentration leading to assymetric peaks and non-reproducible capacity ratios. Eksborg *et al* (136) have shown that in straight phase systems the addition of a hydrophobic ion in sufficient excess will suppress ion-pair dissociation effects, resulting in symmetrical reproducible peaks. Similarly dissociation effects in reversed phase systems are diminished by the presence of competing ions in the mobile phase (134). A further problem with the use of aliphatic alcohols is their known association in aqueous and organic solvents (137-139) making the elucidation of mechanisms and equations difficult.

Karger and co-workers (140, 141) have shown that in straight phase chromatography the retention of sulphonamide tetrabutylammonium ion-pairs decreases with increasing concentration of butan-1-ol in the mobile phase and slopes of -3.4 were found for the log-log plots of capacity ratio against butan-1-ol concentration compared with a value of -2.6 for unionised sulphanilamide. Higuchi *et al* (142) have considered the slopes of such plots to be the average solvation number of the solutes in the organic phase, thus the sulphonamide-tetrabutylammonium ion-pairs are more strongly solvated by butan-1-ol than the unionised sulphanilamide.

(ii) picrate ions:

The picrate pairing-ion system was first introduced into

column liquid chromatography by Eksborg and Persson (143, 144) for the isolation of acetylcholine and choline from rat brain homgenates. Despite being a relatively strong acid, column bleeding of unionised picric acid at low pH is a common problem with picrate columns and becomes serious if it results in pairing-ion depletion or detection interference. Picrate columns also suffer from the disadvantage of the relatively high dissociation constants of picrate ion-pairs in organic solvents.

(iii) alkyl sulphonates:

Two commercial "ion-pair reagents" are available - tetrabutylammonium phosphate and heptanesulphonic acid - and the majority of the applications involving alkylsulphonates have used these commercial materials (see Appendix). One of the most interesting examples of this system is that by Brown *et al*. (145) who separated eight components of an anticholinergic drug formulation and an internal standard.

(iv) hydrophobic ions for the enhancement of detection.

The high UV absorbance or fluorescence of many potentially useful hydrophobic ions (e.g.picrate) make them unsuitable for reversed phase systems. However in a straightphase system if the solute elutes as an ion-pair with a chromophoric pairing-ion, then enhancement of detection in the straight phase mode is possible, and two methods have been described by which detection sensitivity may be enhanced by the use of chromophoric pairing-ions. First, the pairing-ion acts as both the separating and indicating ion. Frei and co-workers (146, 147) found that when using picrate for the separation of plant alkaloids detection limits were increased by a factor of 500. Other examples

of this technique include the use of N,N-dimethylprotriptyline (148) and N-methylimipramine (149) for the detection of carboxylic acids and naphthalene-2-sulphonate (150-152) for the detection of cations.

The second method involves the use of an indicating column loaded with chromophoric ions, and a solute (A) eluting from a first (separating) column as an ion-pair (A,B) exchanges its pairing-ion (B^-) with the chromophoric ion(Z^-) and elutes as a chromophoric ion-pair (A,Z) from the indicating column. Chromophoric ions such as naphthalene-2-sulphonate are often quite hydrophobic and give high extraction constants and therefore have poor separating capabilities, which is an advantage when they are used as indicating ions, since they will not contribute to the separation process and exchange readily with the separating ions (151).

Recently Frei (153) has shown that indicating ions may be used in the aqueous mobile phase in reversed phase systems, provided that a post column extractor which extracts the chromophoric ion-pairs and eliminates the interfering free pairing-ions, is employed.

b. Inorganic pairing-ions

(i) buffer ions:

Buffer ions can act as pairing-ions in their own right as well as contributing to secondary equilibria effects. For example Pryde and Darby (154) separated the quaternary ammonium herbicides paraquat and diquat from biological fluids in a reversed phase system and concluded that the mechanism of retention was due to ion-pair formation

between the solute ions and the phosphate buffer ions (155).

Asmus and Freed (156) compared the separating abilities of a number of inorganic and organic buffer ions and, having found good peak symmetry and column efficiency, concluded that these pairing-ions were a logical and more economical alternative to the more expensive alkylsulphates and sulphonates.

(ii) perchlorate ions:

The use of perchlorate loaded columns stems from their successful use in bulk phase extractions. A number of studies (157, 158) have shown that the specific solvation effects alluded to earlier may be utilised in the optimisation of separations. For example Knox and Jurand (158) compared aliphatic alcohols with methylene chloride and chloroform for the separation of some phenothiazine-perchlorate and dibenzapene-perchlorate ion-pairs. They found that retention decreased with increasing alcohol concentration and decreasing alcohol chain length. Conversely substitution of chloroform by methylene chloride caused a decrease in retention.

c. Surface active pairing-ions

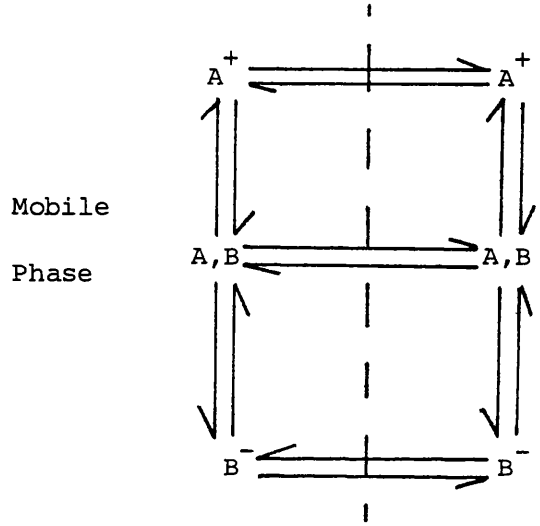
In an attempt to eliminate the problems of peak tailing, column instability and poor reproducibility of many ion-pair systems, two groups independently investigated the use of surface active pairing-ions in HPLC. Haney and co-workers (132, 159) separated a number of tartrazine intermediates using a reversed phase system with tridecylamine and tridecylamine-tributylamine mixtures, as pairing-ions. They argued (132) that retention was due to reversible ion-pair formation in the mobile phase and that separation of the constituents

was on the basis of the different lipophilicities of the formed ion-pairs.

Knox and Laird (120) were at the same time examining different HPLC methods for maximum possible efficiencies. Their work arose out of the problems encountered in the separation of various naphthalenesulphonate derivatives by straight phase HPLC and the very strong polarity of the sulphonate group which caused peak tailing. It occurred to these workers that these problems might be solved by the formation of very stable ion-pairs which would not dissociate and so avoid interaction of the sulphonate group with the silanol groups of the silica. Their choice of cetyltrimethylammonium bromide (CTAB) as pairing-ion and an aqueous alkaline eluent was an excellent one and satisfied their criteria, unfortunately their use of the term "soap chromatography" to describe their approach was not. By convention soaps are long chain carboxylic acid salts of the alkali metals. The term "detergent chromatography"which has been used (160) is also perhaps inappropriate since the surface active molecules are not necessarily acting as detergents.

Knox and Laird found in their initial experiments that while the addition of 1% CTAB improved the adsorption chromatography of the acids on silica the system was not well defined. Therefore they turned to an alkyl silica reversed phase support. In contrast to Haney and co-workers they argued that the mechanism of retention was due to the adsorption of the CTAB onto the stationary phase and an ion-exchange process between the solutes and the adsorbed molecules. One can assume that their initial reason for this assumption was based on the results described earlier by Lederer for an almost identical system in paper chromatography (121).

The different retention mechanisms described by the two original groups have stimulated considerable interest and a number of workers (80, 120, 161, 162, 163) have attempted to elucidate the actual mechanism. The two mechanisms are not mutually exclusive and may be represented by Scheme V



Scheme V

Using a series of dyestuffs and intermediates Knox and Laird (120) showed that in a reversed phase system retention increased with concentration of CTAB in the mobile phase up to 7×10^{-3} mol.dm^{-3} CTAB. Above this concentration of pairing-ion retention of the solutes fell, although the order of elution was unaffected. Knox and Laird concluded that at low concentrations of the CTAB both retention mechanisms were theoretically possible. By investigation of column breakthrough times they found that the CTAB molecules were adsorbed onto the surface of the stationary phase and that over a mobile phase concentration range of 0 to 5×10^{-2} mol.dm^{-3} CTAB a Freundlich type isotherm was obeyed. They calculated that at 5×10^{-2} mol.dm^{-3} CTAB the surface coverage was between 5 and 15%, depending on the "depth" assigned to the alkyl chains on the surface. It is to be assumed that the remaining surface of the stationary phase was covered with adsorbed propan-1-ol from

the eluent. Assuming that the concentration of the solute in the stationary phase ($[B^-]_s$) is negligible compared with $[A,B]_s$, it can be shown that the distribution coefficient of the solute (D_B) is given by

$$D_B = \frac{\alpha^n . \beta K_s [A^+]_m^{0.8}}{1 + K_m [A^+]_s^n} \qquad (39)$$

where n = 1 for monovalent ion-pairs. K_m and K_s are the ion-pair formation constants in the mobile phase and stationary phase respectively and α and β are constants. Equation 39 shows that the distribution constant and hence the capacity ratio will rise with increase in concentration of CTAB. The fall in capacity ratio at higher pairing-ion concentrations was attributed to micellisation of the CTAB which solubilised the solute ions (i.e. acting as a detergent).

The above relationship was not obtained when silica gel was used as a stationary support and there was no evidence of pairing-ion uptake onto the stationary phase. In this mode, Knox and Laird concluded that ion-pairs were formed in the mobile phase and were solvated by the propan-1-ol. These highly solvated ion-pairs were then adsorbed onto the silica surface itself coated with propan-1-ol and which acted as a binder to hold the ion-pairs. No evidence of micellar or clustered CTAB species was found in this mode. Knox and Laird concluded that the solute ions in the reverse phase mode were retained by an ion-exchange mechanism with the pairing-ion though their arguments could be described as specious. Despite the lack of substantial evidence of an *in situ* ion-exchange mechanism this initial work revealed two important considerations. Firstly, it showed that the measurement of adsorption isotherms could yield information about the retention mechanism, and

secondly, it highlighted the importance of the two ion-pair formation constants K_m and K_s. It follows that if K_s is small and K_m is large then the mechanism is ion-pair formation in the mobile phase with subsequent distribution to the mobile phase. Conversely, if K_m is much larger than K_s then ion-pairs will not exist in the mobile phase and an *in situ* ion-exchange is the main mechanism of retention. If K_s and K_m are of similar size then a mixed retention mechanism will exist.

The work of Knox and Laird has been developed further by Scott and Kucera (161) who investigated the retention behaviour of ionic solutes in the presence of alkylsulphonates and quaternary alkylammonium halides using an alkylsilica stationary phase and hydro-organic mobile phases. They found that octanesulphonate was adsorbed onto the stationary phase and the amount taken up was related to the mobile phase concentration according to the simple Langmuir expression. The existence of Langmuir isotherms was confirmed by relating the corrected retention volume of an injected sample of octanesulphonate in a system containing different amounts of octane sulphonate in the mobile phase. Based on found linear relationships between corrected retention volumes and pairing-ion mobile phase concentrations for tetrabutylammonium iodide, sodium hexanesulphonate and sodium octanesulphonate using different reversed phase materials and different mobile phase concentrations of methanol and acetonitrile, Scott and Kucera have concluded that these pairing-ions are adsorbed onto the surface of the alkylsilicas, such that these adsorbed pairing-ions are capable of retaining solutes of opposite charge by an ion-exchange mechanism. Scott and Kucera then related the retention volume of an ionised solute to the retention volume of the adsorbed pairing-ion. Linear relationships were found between solute corrected retention

volume for methylnicotinamide, nicotinoyl alcohol and pyridoxime and pairing-ion concentration for octanesulphonate (<0.3% w/v). At higher concentrations of octanesulphonate the retention volumes of the solutes fell and this was attributed to significant ionic interactions in the mobile phase. Coupled with conductivity and nuclear magnetic resonance studies which confirmed the absence of ion-ion interactions between octanesulphonate and pyridoxime at low methanol concentrations (<50% v/v) and evidence of significant ion-ion interactions at higher methanol concentrations, Scott and Kucera concluded that an *in situ* ion-exchange was the predominant retention mechanism at low pairing-ion concentrations and low organic solvent concentrations, although ion-pair formation in the mobile phase could occur at higher mobile phase concentrations of methanol and with higher concentrations of pairing-ion.

These elegant studies by Scott and Kucera assumed that ion-ion interactions were the primary cause of retention and neglected the possibility of water reinforced ion-pairing (95, 103, 104) in which the dominant effect is solvophobic rather than electrostatic. Furthermore, the tetrabutylammonium ions are not surface active and the charge centres of tetrabutylammonium ion-pairs are considerably further apart than for example CTAB ion-pairs. Horváth and co-workers (162) have considered the effect of charge separation on the retention mechanism as discussed later in this Section.

A similar study to that by Scott and Kucera was performed by Deelder and Linsson (163) who considered the distribution mechanism of amines and amino acids on alkylsilica using alkylsulphate pairing-ions. By measurement of breakthrough times they found that the uptake

followed an L or H isotherm and that the amount adsorbed, X_s, was related to the mobile phase concentration, $[X]_m$, according to the Freundlich equation:

$$X_s = a[X]_m^b \qquad (40)$$

where a and b are constants. Deelder and Linsson found that the uptake of the pairing-ion and the retention of the basic solutes were dependent upon the temperature and the concentration and type of the organic modifier (propan-1-ol and propan-2-ol). The retention of the solute was correlated with the amount of pairing-ion adsorbed and the mechanism was rationalised solely in terms of conventional ion-exchange theory. Equation 20 (Section 1.2.4) may be modified to include the ion-exchange ligand, X_s, which in the present case is the adsorbed pairing-ion, thus the distribution ratio of a solute A^+, D_{A^+} is given by

$$D_{A^+} = K_E . [X^-]_s . [B^+]_m^{-1} \qquad (41)$$

where K_E is the ion-exchange constant and B^+ is the pairing-ion's counter-ion. Hence it follows that:

$$\kappa_{A^+} = K_E . ([X^-]_s . [B^+]_m^{-1}) (V_s . V_m^{-1}) \qquad (42)$$

Therefore it follows that the capacity ratio of the solute is reciprocally related to the concentration of counter-ion in the mobile phase. These workers found that the ion-exchange constant was the dominant term governing retention and was influenced by temperature and the alkyl chain lengths of the pairing-ions. The results presented by Deelder and Linsson assume that ion-exchange is the sole mechanism of retention and rely on the dependence of the capacity ratio on the reciprocal sodium ion concentration. However it should be noted that a similar relationship will exist for an ion-exchange process between ions and formed ion-pairs in the mobile phase. The

most comprehensive phenomenological treatment of these systems has been by Horváth *et al.* who considered that three species could exist, i.e. the pairing-ion, X, the solute, Q, and the formed ion-pair Q,X either free in the mobile phase, m, or bound to the stationary phase ligand, L_s. Hence five important equilibria affecting retention may be defined:

1. Distribution of the ionised solute, K_1.

$$K_1 = [LQ]_s . [Q]_m^{-1} . [L]_s^{-1} \quad (43)$$

2. Ion-pair formation in the mobile phase, K_2.

$$K_2 = [QX]_m . [Q]_m^{-1} . [X]_m^{-1} \quad (44)$$

3. Adsorption of the pairing-ion, K_3.

$$K_3 = [LX]_s . [L]_s^{-1} . [X]_m^{-1} \quad (45)$$

4. Ion-pair distribution to the stationary phase, K_4.

$$K_4 = [LQX]_s . [L]_s^{-1} [QX]_m^{-1} \quad (46)$$

5. Ion-exchange, K_5.

$$K_5 = [LQX]_s . [LX]_s^{-1} [Q]_m^{-1} \quad (47)$$

The distribution ratio of the solute, D_Q, is given by:

$$D_Q = ([LQX]_s + [LQ]_s)([Q]_m + [QX]_m)^{-1} \quad (48)$$

Thus the capacity ratio of the solute is given by:

$$k_Q = D_Q . V_s . V_m^{-1} = V_s . V_m^{-1} ([LXQ]_s + [LQ]_s)([Q]_m + [QX]_m)^{-1} \quad (49)$$

Assuming at equilibrium that the pairing-ion concentration in the mobile phase is constant and well in excess of the solute concentration

then $$[X] = [X]_m \quad (50)$$

similarly it may be assumed that:

$$[L]_{total} = [L]_s + [LQ] \equiv [L] \quad (51)$$

Equations 43-49 may be formalised such that, independent of retention mechanism, the capacity ratio of the solute is related to the pairing-ion concentration

$$\kappa_Q = (\kappa^o + B.[X^-])(1 + K_2[X])^{-1}.(1 + K_3[X])^{-1} \quad (52)$$

where κ^o is the capacity ratio of the solute in the absence of any pairing-ion and B is a constant equal to K_3K_5, if the mechanism is ion-exchange, or K_2K_4, if the mechanism is ion-pair formation in the mobile phase followed by distribution to the stationary phase. If either $K_2[X]$ or $K_3[X]$ are much less than one then Eq. 52 reduces to

$$\kappa_Q = (\kappa^o + B[X])(1 + P[X])^{-1} \quad (53)$$

where P equals K_2 or K_3.

By this treatment Horváth *et al* . indicated that the mechanism of retention cannot be elucidated merely by the investigation of the effect of pairing-ion concentration on retention. However they suggested that the mechanism could be elucidated by the application of Solvophobic Theory (89) to the various equilibria described by Eqs. 43-47.

Equation 17 which describes the behaviour of solute molecules in terms of the rubric of the Solvophobic Theory may be simplified to:

$$\ln\kappa = k_1 - k_2 + k_3.\Delta A \quad (54)$$

where k_1, $k_2$and k_3 are constants depending on the properties of the solvent and the stationary phase. k_2 also depends on the solute dipole, polarisability and molecular volume. ΔA is the difference between the molecular surface area of the solute-hydrocarbonaceous ligand, and that of the solute and that of the stationary phase ligand, such that

$$\Delta A = A_{QL} - A_Q - A_L \quad (55)$$

For an ionised solute from Eqs. 43 and 54 it follows that

$$\ln K_1 = k_1' + k_2 . f(Z) + k_3 . \Delta A \qquad (56)$$

where k_1' has the same meaning as k_1 but excludes the phase volume ratio term, and the function f(Z) is related to the absolute value of the product of the charges on the solute ion and its associated counter-ion.

If the ion-pairing constant (K_2) is due solely to electrostatic interactions then the energy of interaction and hence the equilibrium constant is dependent on the product of the charges, i.e.:

$$\ln K_2 = f'(Z_Q . Z_X) + \text{constant} \qquad (57)$$

and

$$\ln K_5 = f''(Z_Q . Z_X) + \text{constant} \qquad (58)$$

According to Solvophobic Theory both K_3 and K_4 (Eqs. 45 and 46) may be expressed as a function of the decrease in molecular surface area on binding to the stationary phase ligand, i.e.

$$\ln K_3 = k_1' + k_2 \, f(Z_X) + k_3 \, \Delta A_3 \qquad (59)$$

and

$$\ln K_4 = k_1' - k_2 + k_3 \Delta A_4 \qquad (60)$$

ΔA_3 and ΔA_4 may be defined as

$$\Delta A_3 = A_{XL} - A_X - A_L \qquad (61)$$

$$\Delta A_4 = A_{XQL} - A_L - A_{XQ} \qquad (62)$$

The difference between Eqs. 59 and 60 is the function $f(Z_X)$ which arises from the fact that the ion-pair is neutral whereas the pairing-ion is charged.

Defining an enhancement factor (η) as the ratio of the equilibrium constant of the solute between the mobile and stationary phases in the presence and absence of a pairing-ion, then for an ion-exchange process:

$$\ln\eta = \ln(K_5 . K_1^{-1}) \quad (63)$$

and

$$\ln\eta = \text{constant} + f(Z_Q Z_X) - f(Z_X) - k_3(A_{LXQ} - A_{LX} - A_{LQ}) \quad (64)$$

For an ion-pair distribution mechanism the enhancement factor is then given by:

$$\ln\eta = \ln(K_4 . K_1^{-1}) \quad (65)$$

and

$$\ln\eta = \text{constant} - f(Z_Q) + k_3(A_{QXL} - A_{QX} - A_Q - A_{LQ}) \quad (66)$$

Horváth *et al.* (162) showed that $\ln\eta$ was linearly related to the number of carbon atoms in the alkyl chain of the pairing-ion, that is - the enhancement factor is dependent upon the hydrocarbonaceous surface area of the pairing-ion. This relationship can only be satisfied by Eq. 66 which relates the enhancement factor to the pairing-ion surface area. Therefore these workers concluded that ion-pair formation in the mobile phase with distribution to the stationary phase is the mechanism of retention. Equation 64 predicts that for an ion-exchange process to occur the enhancement factor is dependent on the pairing-ion charge .

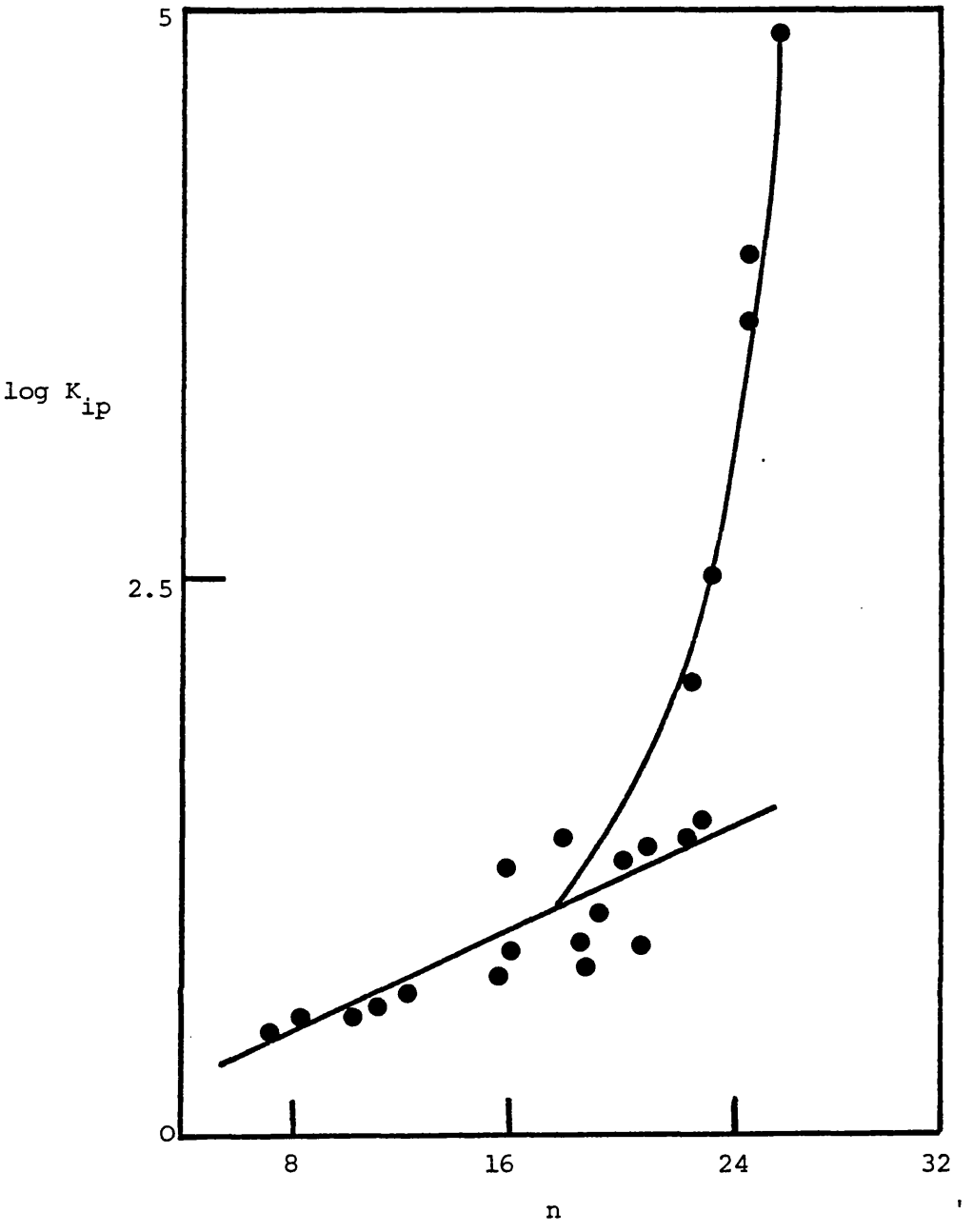
The results presented so far suggest that either mechanism can dominate and neither is mutually exclusive. *A priori* ion-pair distribution can dominate at high pairing-ion concentrations and in mobile phases with a high organic solvent concentration, whereas the ion-exchange mechanism can occur at low pairing-ion concentrations and in solvents of higher dielectric.

The majority of pairing-ions investigated with respect to retention mechanisms have used either non-surface active pairing-ions

(e.g. tetrabutylammoniums or small alkyl sulphates) or relatively hydrophilic surfactants (e.g. octanesulphate). Horváth has suggested that the ion-exchange mechanism can dominate if a hydrophobic non-surface active pairing-ion such as tetrabutylammonium is used, since relatively the charge centres of the ion-pairs are separated by a larger distance and K_2 is smaller. Horváth *et al.* have suggested further that a change in dominant mechanism occurs when K_2 equals K_3 and that as the hydrophobicity of the pairing-ion increases the mechanism changes from ion-pair distribution to ion-exchange. This conclusion was based on the assumption that K_3 increases faster than K_2 with increasing alkyl chain length of the pairing-ion. For surface active pairing-ions Horváth *et al.* calculated that K_3 would be significantly greater than K_2 when the alkyl chain contained 22 carbon atoms. However this assumption was based on the premise that ion-pair formation is due primarily to electrostatic interactions and ignored the effect of water structure reinforced ion-pairing (103). Figure 1.1 shows the relationship between the ion-pair formation constant and the number of carbon atoms in the formed ion-pair (from ref. 104). Above a total carbon number of about 20 the ion-pair formation constants rise much more steeply due to the influence of water structuring.

Kraak and co-workers (164-167) have used both anionic and cationic pairing-ions for the resolution of ionised solutes in the reversed phase mode and chose chromatographic conditions such that the ion-exchange mechanism would be dominant. They termed such systems "solvent generated ion-exchangers". In some cases it was found that column stability was maintained after the column had been pre-loaded with a high surfactant concentration either by the use of a pre-column in the absence of any surfactant in the mobile phase, or by the use of

Fig. 1.1. The relationship between the total number of carbon atoms in an anion-cation ion-pair, n, and their association constants, K_{ip}: (ref. 104).



very low mobile phase concentrations of surfactant. The fact that a column previously equilibrated with a high concentration of surfactant is stabilised by a mobile phase containing a very low concentration of pairing-ion is a surprising one, but must reflect the strong binding affinities of the pairing-ions.

Kraak and co-workers have described the use of solvent generated cation-exchangers for the resolution of amino acids, peptides and catecholamines (164-166) and solvent generated anion-exchangers for the resolution of aromatic acids (167). In agreement with the later work of Deelder and Linssen (163) they found that the capacity ratio of cationic solutes was linearly related to the reciprocal of the sodium ion concentration when an anionic surfactant was employed at low pH.

Kraak *et al.* (167) found that stable systems could be produced with no pairing-ion present in the mobile phase. If a high concentration of a pairing-ion was maintained in the mobile phase after equilibration of the column, it was found that retention was lower than when the pairing-ion was removed. This reduction in retention was attributed to micellisation of the pairing-ion with distribution of the solute ions into the eluent micelles. Equation 52 predicts that retention can fall at higher sub-micellar concentrations of pairing-ion, consequently the effect described by Kraak attributable to micellisation is an additional phenomenon not accounted for by Horváth *et al.*'s treatment (167), although it should be pointed out that Kraak *et al.* did not, in fact, experimentally determine any micellisation in their systems.

1.5 Linear Free Energy Relationships

Hammett (168) first suggested an equation of the form

$$\log (k.k_o^{-1}) = \rho\sigma \qquad (67)$$

might be employed to correlate the influence of 3- and 4- substituents on the reactivity of substrates containing aromatic groups. k and k_o are the reaction rate constants of the substituted and reference compounds respectively, σ and ρ were defined as the substituent and reaction parameters respectively. Equation 67 is now recognised as an example of a linear free energy relationship (169) which are regarded as linear relationships between the logarithms of the equilibrium (or rate) constants for one reaction series (K^B) and those for a second reaction series (K^A) subjected to the same variation in reactant structure or reaction conditions, i.e.

$$\log K^A = m \log K^B + c \qquad (68)$$

where m and c are the slope and intercept of the obtained relationship. The logarithm of the equilibrium constant (or rate constant) may be related to the standard free energy change (or standard free energy of activation), ΔG^o accompanying the reaction:

$$\log K = - \Delta G^o (2.303.RT)^{-1} \qquad (69)$$

Thus it follows that:

$$\Delta G^B = n\Delta G^A + d \qquad (70)$$

and the empirical correlation (Eq. 68) is equivalent to the linear free energy relationship (Eq. 70).

Using the extrathermodynamic approach of Leffler and Grunwald (170) it is possible to utilise substituent parameters obtained from model systems (e.g. partition studies) to evaluate substituent effects in other systems (e.g. biological activity, chromatography, etc.). An extrathermodynamic approach may be described as one using relation-

ships not directly resulting from the principles of thermodynamics, although the actual approach resembles that of thermodynamics in the sense that detailed mechanisms need not be explicitly identified (171).

A decade ago Higuchi and Davis (172) compiled a list of some 40 linear free energy correlation substituent parameters, of these the most widely used were (and still are), the liquid-liquid distribution coefficient, log K_D, (173), the Hansch hydrophobic bonding constant, π, (174), obtained from octan-1-ol:water liquid distribution studies, the Hammett electronic term, σ, (168) and the Rm and ΔRm terms obtained from reversed phase thin-layer chromatography (175). Clearly the τ value described in Section 1.2.2.c constitutes another extrathermodynamic substituent parameter.

1.5.1 Biological activity

The most comprehensive use of linear free energy relationships for the correlation of biological activity with molecular structure has been initiated by Hansch and Fujita (173, 174) whose approach has been to factor the effects of substituents on the rate or equilibrium constants into free energy based terms. Thus the biological response of a derivative, x, may be given by:

$$\log BR_x = \log C_x^{-1} = k\pi + \rho\sigma + k'S + k'' \qquad (71)$$

where C_x is the molar concentration of the derivative, x, having a standard biological response (e.g. the minimum inhibitory concentration of an antibiotic). π, σ and S are extrathermodynamic hydrophobic, electronic and steric terms respectively and k, k' and k'' are the constants of the regression analysis.

1.5.2 Chromatography

Functional group analysis and linear free energy relationships have been applied widely in chromatography, since chromatographic retention may be related to the equilibrium constants of solutes between the mobile and stationary phases. This approach has been adopted to generate functional group values for use in non-chromatographic areas (e.g. prediction of biological activity) or for the rationalisation of retention behaviour.

In chromatographic systems employing aqueous and organic phases it is reasonable to assume that group values will be determined predominantly on the basis of hydrophobicity. Consequently, chromatographic functional group values have been used widely in the correlation analysis of biological activity and this subject has been critically reviewed by Tomlinson in 1975 (176).

The theoretical basis for the relationship between the R_F value in liquid-liquid chromatography and chemical structure was proposed by Consden *et al* (177) and Martin and Synge (9) who deduced that for ideal solutions the partition coefficient of a solute Q, between the two phases, K_{D_q}, was related to the free-energy required to transport one mole of Q, $\Delta\mu_Q$, from one phase to another by:

$$\ln K_{D_Q} = \Delta\mu_Q (RT)^{-1} \qquad (72)$$

Martin and Synge (9) showed that the addition of a group x to the substance Q would change the partition coefficient by a factor depending on the nature of x and the two phases, but not Q itself. Hence if Q is substituted by n groups x and m groups y to give C, then:

$$RT \ln K_{D_C} = \Delta\mu_Q + n\Delta\mu_x + m\Delta\mu_y. \qquad (73)$$

The partition coefficient may be related to the R_F by

$$K_{D_Q} = A_m.A_s^{-1}(R_F^{-1} - 1) \quad (74)$$

where $A_m.A_s^{-1}$ is the effective ratio of the cross-sectional areas of the mobile and stationary phases. Substitution of Eq. 74 in Eq. 73 gives

$$RT \ln A_m.A_s^{-1}(R_F^{-1}-1) = \Delta\mu_Q + n\Delta\mu_x + m\Delta\mu_y \quad (75)$$

Bate-Smith and Westall (175) introduced the R_M term, where R_M equals $\log(R_F^{-1} - 1)$. The effect of the substituent, x, on the partition coefficient may be given by (174):

$$\log(K_{D_j}.K_{D_i}^{-1}) = \pi_x k_p \quad (76)$$

where k_p is a constant for a particular phase system.

If k_p is unity, then:

$$\pi_x = \log K_{D_j} - \log K_{D_i} \quad (77)$$

and

$$\Delta(\Delta G^o)_x = \Delta G_j^o - \Delta G_i^o \quad (78)$$

Thus:

$$\Delta(\Delta G^o)_x = RT \ln K_{D_i} - RT \ln K_{D_j} \quad (79)$$

or:

$$\ln K_{D_j} - \ln K_{D_i} = -\Delta(\Delta G^o)_x(RT)^{-1} \quad (80)$$

$$\log (K_{D_j}.K_{D_C}^{-1}) = k(-\Delta(\Delta G^o)) = \pi_x \quad (81)$$

where k is a grouped constant. Substitution of Eq. 77 in Eq. 74 gives (176):

$$\pi_x = \log A_m.A_s^{-1} + \log(R_{F_X}^{-1}-1) - \log A_s.A_m^{-1} + \log(R_{F_i}^{-1}-1) \quad (82)$$

which in terms of R_m(175), becomes:

$$\pi_x = R_{M_j} - R_{M_i} \quad (83)$$

and hence we can show, with the above assumptions, that:

$$\pi_x = \Delta R_{M_x} \quad (84)$$

Therefore ΔR_M values are analogous to π values and may be used either to predict bulk phase partition substituent values or more usefully to predict biological activity directly.

Clearly R_M values will depend upon the nature of the two phases and Collander (178) has shown that the partition coefficients of solutes in one phase system, K_{D_1}, are related to the partition coefficients in a second system by the linear equation:

$$\log K_{D_1} = a \log K_{D_2} + b \qquad (85)$$

provided that the polar phase is water and the organic phases contain the same functional groups. Dearden and Tomlinson (179) obtained good correlations between ΔR_M values obtained in different phase systems for a series of substituted acetanilides.

An important principle of hydrophobicity indices based on π, ΔR_M or τ values is that of additivity, i.e.

$$R_{M_C} = R_{M_Q} + \Delta R_{M_x} + \Delta R_{M_y} + \ldots. \qquad (86)$$

and

$$\log K_{D_C} = \log K_{D_Q} + \pi_x + \pi_y + \ldots \qquad (87)$$

From the point of view of prediction it is important that ΔR_M or π values are constant and independent of the phase system in which they were determined. In practice factors such as electronic and steric effects can influence hydrophobic fragmental constants and a number of workers (180-183) have attempted to rationalise these effects and to produce self-consistent hydrophobicity indices.

1.5.3 High performance liquid chromatography

Extrathermodynamics (linear free-energy relationships, entropy/enthalpy compensation etc.) have been used by many workers using HPLC systems. Such approaches have been used in general respects to examine retention data obtained upon varying experimental and constitutional conditions, i.e. a self-consistent approach; as well as in more specific instances, for example in relating reversed phase HPLC parameters to bulk phase liquid-liquid distribution parameters so as to help elucidate retention mechanisms or conversely to use reversed phase HPLC parameters to generate information on the hydrophobicity (*sic*) of solutes or functional groups.

Examining first these specific uses of extrathermodynamics, it is useful to this present study to describe the work of Yamana *et al.* (184) who investigated a reversed phase system for the development of a lipophilicity index based on log κ values for a series of penicillins and cephalosporins. Due to the high hydrophobicity of many of the solutes a purely aqueous mobile phase was not always practicable. However, they found that the capacity ratio of the solutes in pure water, κ_o, could be determined by extrapolation of Eq. 88, i.e.

$$\log \kappa = a.\phi + \log \kappa_o \qquad (88)$$

where a is a regression coefficient and ϕ is the concentration of organic modifier, (methanol),in the mobile phase. Good correlations were obtained between log κ_o and both the solutes' octan-1-ol/water liquid-liquid distribution coefficients and their R_M values, obtained using either octan-1-ol or silicone oil as the reversed phase. Yamana *et al*. concluded that since for such relationships slope coefficients of approximately one were obtained, then log κ_o values could be used as a standard reference system. Unfortunately they did not define adequately the stationary phase material used. From a

practical point of view they pointed out the tedious nature of the extrapolation technique and developed a second index based on capacity ratio measurements with a mobile phase of 30% v/v methanol; and although good linear relationships between log κ and log K_D and R_M were obtained, the presence of methanol in the mobile phase was reflected in higher slope coefficients. The dangers inherent in such extrapolation techniques are pointed out in the Discussion Section of this thesis.

In contrast to Yamana who used an octadecyl-silica stationary phase, Mikaye and Terada (185) have described the use of a 1% octanol stationary phase coated *in situ* onto silica. Using a purely aqueous mobile phase and simple aromatic solutes they obtained linear relationships (r more than 0.992) with slopes close to unity for the relationship

$$\log K_{D_{octanol}} = \log K_{D_{HPLC}} + b \qquad (89)$$

where

$$\log K_{D_{HPLC}} = \log \kappa - \log(V_s.V_m^{-1}) \qquad (90)$$

Prior to the work of Mikaye and Terada, other groups (186-191) had attempted to produce high performance liquid chromatography systems with "octanol:water"-like properties. Henry *et al.* (186) obtained good correlations between the logarithm of the retention volumes and log K_D for a series of sulphonamides and barbiturates, using purely aqueous mobile phase and octadecylsilica stationary phase. However, they obtained poor correlations when stationary phases of silica coated with squalene (r=0.294) and octanol (r=0.863) were used. These poor correlations were due to poor coating of the liquid stationary phase resulting in significant interactions of the solutes with the silica surface.

Taylor and co-workers (187) similarly described a liquid chromatography method for the determination of partition coefficients to be used in drug design models. Octanol saturated with water was coated onto kieselguhr and a mobile phase of water saturated with octanol was used as the eluent. For a number of heterocyclic compounds and β-adrenergic receptor blockers the following relationship was obtained:

$$\log t_c = 1.006 \log K_D - 0.622 \qquad \begin{matrix} n = 7 \\ r = 0.99 \end{matrix} \qquad (91)$$

where, $t_c = t_r - t_o = \kappa . t_o$.

Taylor and co-workers indicated the problems encountered by Yamana *et al.* that very hydrophobic solutes are uneluted or at best, have very long retention times in reversed phase systems. The maximum measurable range of log K_D using Taylor's method is -0.3 to 3.7.

For ion-pair systems Eqs. 32-36 show that the distribution ratio, D, may be determined by simple chromatographic measurements. Equation 92 shows the relationship between the distribution ratios obtained for amine-perchlorate ion-pairs measured by both HPLC and bulk-phase extraction, as derived from ref. 141.

$$D_{HPLC} = 0.90\ D_{BULK} + 0.26 \qquad (92)$$

The correlation coefficient of 0.997 indicates that about 1% of the variance in the correlation is unexplained by the relationship, which suggests that bulk phase extraction data may be used to predict retention in ion-pair chromatography (or vice versa). However, good correlations between chromatographic distribution constants and bulk phase ion-pair extraction data often do not exist due to the difficult experimental factors (e.g. mutual phase saturation), and interfering

secondary equilibria (140).

The measurement of partition coefficients by high performance liquid chromatography assumes that the solute partitions between the two phases. This assumption should be viewed within the context of the comments in Section 1.2.4b regarding partitioning in liquid-liquid chromatography.

The use of relative retention data based on functional group differences has been used in high performance liquid chromatography to rationalize retention behaviour, to investigate solute-solvent interactions and to predict retention within a series of compounds.

Frei and co-workers (192) have investigated two phenothiazines (thioridazine and northioridazine) and their corresponding oxidation (degradation) products using straight phase liquid-solid chromatography. They identified the particular functional groups responsible for selectivity within the two series. While the capacity ratios of the pairs of compounds in the two series varied considerably due to their different basicities, the α values for the functional groups were in good agreement. Consequently, they were able to construct elutropic diagrams based on α values to predict the chromatographic behaviour of the oxidation products of new phenothiazines.

Karger *et al.* (140) used relative retention data to optimize the separation of primary and secondary biogenic amines in a perchlorate straight phase ion-pair system. By examining α values they were able to obtain good separations for primary and secondary biogenic amine using relatively weak solvating components such as butan-1-ol or ethyl acetate

in the mobile phase. However, between normetanephrine-metanephrine and noradrenaline-adrenaline little separation could be achieved unless a very basic organic modifier (tributylphosphate) was used, which was attributed to the greater hydrogen bonding capacity of the primary amines resulting in greater solvation of the formed perchlorate ion-pair, and elution of the primary amine before the secondary amine.

Molnár and Horváth (193) have used a functional group approach to rationalise the separation of some 32 catecholamines and related compounds in a simple reversed phase system. An octadecyl silica column (LiChrosorb RP18) was used, with a purely aqueous phosphate buffer (pH 2.1) as mobile phase. They found it convenient to obtain capacity ratios by raising the temperature of the system to 70°C, rather than by employing an aqueous-organic mobile phase. At the pH of the mobile phase, ionisation of these solutes' carboxyl groups was suppressed, and their amino groups were fully protonated. Retention and selectivity was rationalised by these workers in terms of Solvophobic Theory such that the effect of a substituent can be shown to be dependent on its relative effect on k_2 and ΔA (Eq. 54, Section 1.4.3 ϕ. Addition of a polar group (e.g. OH or NH_3^+) reduces retention due to an increase in the value of k_2, whereas addition of an "unpolar" or hydrophobic group (e.g. replacement of OH with OCH_3) increases retention due to an increase in the hydrocarbonaceous surface area of the solute (i.e. an effect on ΔA). In the majority of cases constant log α, (i.e. τ), values for the replacement of H with a particular functional group were found. Inconsistent values observed for τ were due to steric effects or differences in the ionisation constant. However notwithstanding such observations these workers concluded that functional

group values so obtained were analogous to the Hansch π values and have potential in quantitative structure-activity relationships. The data obtained by Molnár and Horváth using LiChrosorb RP-18 columns were compared later (194) to data obtained using two different stationary phases, under operating conditions such that each stationary phase was examined at a temperature different than the others. After analysis of their results in terms of various extra-thermodynamic relationships these workers concluded that selectivity differences were not due to differences in stationary phase carbon loading but solely due to different operating temperatures.

Karger *et al.* (195) have used Solvophobic Theory to investigate the effects of organic solvents on hydrophobic (86) and polar (195) functional group selectivity in reversed phase systems. The methylene increment (α_{CH_2}) for a series of n-alcohols was used as a model for hydrophobic selectivity. They found that hydrophobic selectivity decreased with increasing mobile phase concentration of methanol, propan-1-ol, acetonitrile and acetone. However using time normalisation conditions it was found that hydrophobic selectivity was independent of the organic modifier type. However when a series of ketones, esters, primary and secondary alcohols were compared they found that polar selectivity was not constant under conditions of time normalisation. These results may be explained in terms of Solvophobic Theory. In selectivity terms Eq. 54 may be written as:

$$\tau = -\Delta k_2 + k_3.\Delta(\Delta A) \qquad (93)$$

where Δk_2 is related to the free energy of interaction of the functional group with the mobile phase due to van der Waal's interactions, k_3 is a constant related to the surface tension of the mobile phase and $\Delta(\Delta A)$ is the change in hydrocarbonaceous surface area of the solute

due to the substituent, and is approximately equal to the hydrocarbonaceous surface area of the group (196). Hence polar selectivity will be influenced by both terms in Eq. 93 and hydrophobic selectivity will be dominated by the second term, which may be represented by $f\gamma.\Delta(\Delta A)$ since γ is proportional to k_3 (87).

The effect of two organic solvents on selectivity may be compared by considering Eq. 93 further, i.e.:

$$\tau^1 - \tau^2 = -\Delta k_2^1 + \Delta k_2^2 + f\gamma^1\Delta(\Delta A) - f\gamma^2\Delta(\Delta A) \qquad (94)$$

where superscripts 1 and 2 refer to two mobile phases 1 and 2.

Karger *et al*. (86) have shown that if γ^1 equals γ^2 then the hydrophobic content of the selectivity is normalised. Under such conditions we may now write:

$$\tau^1-\tau^2 = \Delta k_2^2 - \Delta k_2^1 \qquad (95)$$

and for time normalisation and hydrophobic normalisation conditions then:

$$\tau^1-\tau^2 = \Delta k_2^2 - \Delta k_2^1 = \log\ (\kappa_j^1/\kappa_j^2) \qquad (96)$$

where j, as before, refers to a substituted compound.

Hence differences in polar selectivity of organic solvents are due to their different solute-solvent interactions, i.e. v an der Waals interactions. Karger *et al*. (195) also compared the retention of some 28 mono- and di-substituted phenyl derivatives substituted with polar or hydrophobic moieties using hydrophobic normalisation with different mobile phases containing 'equivalent' concentrations of methanol, tetrahydrofuran and acetonitrile in the eluent. Here mobile phase equivalency was taken as the concentration of organic modifier giving a mobile phase surface tension of approximately 34.5 $mN.m^{-1}$. Analysing

their data using Eq. 97, i.e.:

$$\log \kappa = m.\log K_D + c \qquad (97)$$

where K_D is the octan-1-ol: water liquid-liquid distribution coefficient, they found, with the exception of phenolic compounds, good linearity with a slope of 0.60 when methanol was the organic modifier. The deviation in the phenol values from the general relationship was explained in terms of Davis's observation (197) that phenols can hydrogen bond with octan-1-ol, such that the measured K_D value is not due solely to a hydrophobic effect. Lower slopes and poorer correlations of log κ with log K_D were obtained for THF and acetonitrile mobile phases due to significant deviations of the polar substituted solutes. The different organic modifiers were then compared using Eq. 98.

$$\log \kappa^1 = a\log \kappa^2 + c \qquad (98)$$

Solutes containing purely hydrophobic groups as expected lay on the calibration line with a slope of unity. Again significant deviations from the calibration line were obtained for the polar substituted solutes especially when the organic modifier used was THF.

A similar study was performed by Bakalyer *et al.* (198) who investigated eleven functional groups mono substituted into three reference solutes, benzene, butylbenzene and naphthalene. Both binary and ternary mixtures of methanol, acetonitrile and THF in water were investigated. Significant polar selectivity differences of the three organic modifiers were obtained and these differences were rationalised in terms of specific solute-solvent interactions such as dipole interactions and hydrogen bonding. Interestingly Bakalyar *et al* . found that the polar selectivity depended upon the chosen reference solute. This arose because the hydrophobic content of selectivity was not normalised,

i.e. the conditions were not chosen such that the selectivity factor for benzene/butylbenzene and benzene/naphthalene was constant. If hydrophobic normalisation is not employed Eq. 98 is not strictly relevant for the comparison of polar selectivities.

1.6 Scope of this Thesis

The use of large hydrophobic pairing-ions in reversed phase HPLC for the resolution of ionised solutes is now well established. Despite the wide application of these systems, many of the physicochemical principles governing retention and selectivity are less well understood. Many studies (80, 120, 161, 163, 199) on the mechanism of retention in these systems have involved the correlation of retention with the stationary phase charge density resulting from adsorption of the pairing-ion, and have tended to ignore those interactions occurring in the mobile phase (162).

Other workers have demonstrated that a study of functional group behaviour and the application of linear free energy relationships (193-195, 198) and Solvophobic Theory (29, 86-88, 196) can yield information on the mechanism(s) of retention for neutral solutes in reversed phase HPLC. Hence it is the purpose of this present study to investigate the behaviour of functional groups in reversed phase ion-pair systems,since only few reports on such behaviour exist in the literature (200, 201). In addition to revealing information about the retention mechanisms, the investigation of functional group behaviour and the application of linear free energy relationships was undertaken to determine whether such approaches may be used for the prediction of retention and selectivity in such systems, and if the information obtained can be used in describing drug

properties in other (non-chromatographic) areas such as drug design.

Model systems of cationic and anionic solutes have been used, with homologous series of alkylbenzyldimethylammonium chloride and alkylsulphates as pairing-ions. Micellisation of the pairing-ion is considered to affect retention in reversed phase ion-pair systems (120, 165) and hence sub-micellar concentrations of pairing-ions have been used throughout. The ABDACs are particularly suited to this type of study since their physicochemical properties are well documented (95, 104, 202). Consequently, the primary model system investigated was a series of mono-substituted benzoic acids, which can be fully ionised within the pH limits (2-7.5) of reversed phase systems, and an homologous series of ABDAC pairing-ions. Additional functional groups of wider physicochemical character have been examined using a series of 1,3,5-s-triazines, (paired with SDDS) and a series of 8-azapurin-6-ones (paired with C_{11}BDAC).

Retention and selectivity in these systems is considered to be affected by a larger number of environmental and constitutional factors such as pairing-ion type and concentration, ionic strength, stationary phase composition and organic modifier type and concentration all of which have been investigated here. In addition the effect of temperature on solute and group behaviour has been examined so as to obtain information about the thermodynamics of the chromatographic process.

This present study has been restricted to an investigation of hydrophobic surface active pairing-ions, and the results obtained here have been related to those obtained in other studies which have used

either surface active pairing-ions (e.g. ref. 120) or non-surface active pairing-ions (e.g. refs. 80 and 161).

The derived theories have been tested by their application to problems of pharmaceutical and biomedical interest such as antibiotic drug design and separation of drugs from structurally related compounds, degradation products and metabolites. No reports exist in the literature realising the prediction of Krull (203) that ion-pair techniques may be applied to HPLC systems for the resolution of optical isomers and therefore a preliminary investigation into this area was made, laying some of the ground rules for future studies.

This thesis is organised such that the bulk of the experimental results obtained have been combined into one section (Results, Section 3), and these results are referred to in the Discussion section (Section 4).

SECTION 2. EXPERIMENTAL

SECTION 2 EXPERIMENTAL

2.1 Materials

2.1.1 Solvents

HPLC grade solvents were used throughout without further purification. Methanol (MeOH), tetrahydrofuran (THF) acetonitrile (MeCN) and hexane were from Rathburn, Peebleshire, U.K. The water used for the preparation of all mobile phases was freshly double distilled from an all glass still. For the effect of ionic strength investigation and the determinations of critical micelle concentration the water was deionised by passage through an Elgastat deioniser (Elga Products, Buckingham, U.K.).

2.1.2 Acids, bases and buffer salts

Several acids, bases and buffer salts, all of Analar grade, were obtained from two sources. K_2HPO_4, NaH_2PO_4, Na_2HPO_4, NH_3(880), HNO_3 H_2SO_4, and $ZnSO_4$ were from Fisons, H_3PO_4, HCl, KNO_3 and di-sodium hydrogen citrate were from BDH Ltd., Poole, U.K.

2.1.3 Surface active pairing-ions

Homologous series of cationic surfactants (alkylbenzyldimethylammonium chlorides - ABDACs) were obtained from either Fluorochem or Sterling-Winthrop as have been described elsewhere (202). Anionic surfactants (alkylsulphates) were of at least Analar grade and were used as received from various sources. The molecular weights and sources of these surfactants are listed in Tables 2.1 and 2.2.

The metal chelate surfactant (12-dien-Zn(II)) was prepared from zinc sulphate and dodecyldiethylenetriamine (ex. Kodak Ltd., Liverpool,

U.K.) as described in Section 2.3.10. Two optically active pairing-ions were employed, D-camphor-10-sulphonic acid was obtained from Sigma, Poole, Dorset, U.K. (Sigma grade) and D,L-carboethyoxyheptyl-trimethylammonium methanesulphonate was synthesized from 2-amino-octanoic acid (Section 2.3.11). The structures of the pairing-ions are given in Fig. 2.1.

2.1.4 Solutes

Several solutes were obtained from a variety of sources and used as received. These solutes were of at least reagent grade and are listed (with their sources) in Tables 2.3 to 2.6 under the general classes: benzoic acid derivatives, phenylalanine metabolites, tryptophan metabolites and histidine metabolites. A sample of sodium cromoglycate (SCG) was donated by Fisons, Loughborough, U.K.

A number of solutes were obtained from non-commercial sources. Propranolol, practolol, atenolol and related compounds, chlorthalidone, bendrofluazide and their degradation products, 4-hydroxyphenylglycine (4-HPG), 2-hydroxyphenylglycine (2-HPG), sodium 4-hydroxymandelate (SHM) and 6-chlorocinnoline-3-carboxylic acid (CCA) were donated by Imperial Chemical Industries, Macclesfield, U.K. (Mr. P.N. Brittain). ICI also supplied three peptides carbobenzoxy-tyr-ser.leu, glu-his.trp.ser.NH.NH_2 and arg.pro-azaglycine amide and their synthetic starting materials and samples of 'Tenoretic' tablets and 'Inderetic' capsules.

Substituted 8-azapurin-6-ones and substituted 1,3,5-s-triazines were donated by May and Baker, Dagenham, U.K. (Dr. K.R.H. Wooldridge). The structures of these solutes are shown in Figs. 2.2a to 2.2n.

Table 2.1 Homologue numbers, molecular weights, purities and sources of the alkylbenzyldimethylammonium chloride pairing-ions

ABDAC	Homologue number	Purity	Molecular[1] weight	Source
C_8BDAC	8	2	301.9	Sterling-Winthrop[3] New York
C_{10}BDAC	10	2	330.0	Sterling-Winthrop[3] New York
C_{11}BDAC	11	2	344.0	Sterling-Winthrop[3] New York
C_{12}BDAC	12	2	358.0	Sterling-Winthrop[3] New York
C_{13}BDAC	13	2	372.0	Sterling-Winthrop[3] New York
C_{14}BDAC	14	puriss[4]	386.1	Fluorochem,Glossop
C_{16}BDAC	16	puriss[4]	414.1	Fluorochem, Glossop

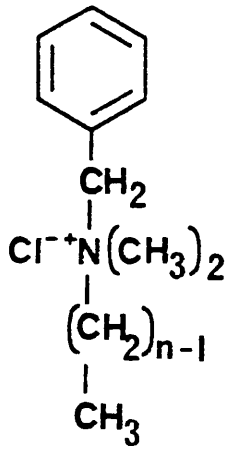
1. Monohydrate
2. Ref. 202
3. Gift
4. More than 99%

Table 2.2 Homologue numbers, molecular weights, purities and sources of alkylsulphate pairing-ions

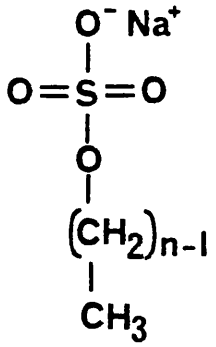
Alkylsulphate	Homologue number	Purity	Molecular weight[1]	Source
Sodium octyl-sulphate (SOS)	8	>99%	232.2	Cambrian Chemicals, Croydon
Sodium decyl-sulphate (SDS)	10	>99%	260.4	Cambrian Chemicals, Croydon
Sodium dodecyl-sulphate (SDDS)	12	specially purified for bio-chemical work	288.4	BDH, Poole
Sodium tetra-sulphate (STDS)	14	>99%	316.5	Cambrian Chemicals, Croydon

1. Anhydrous

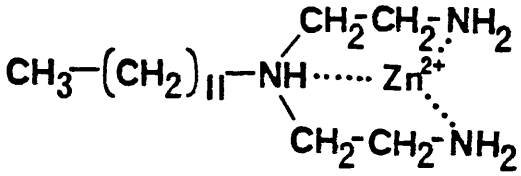
Fig. 2.1 The structure of the pairing-ions



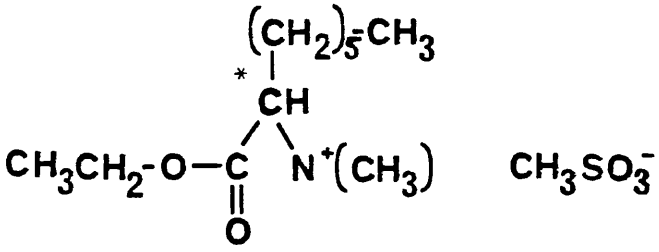
Alkylbenzyldimethylammonium chlorides (ABDACs)



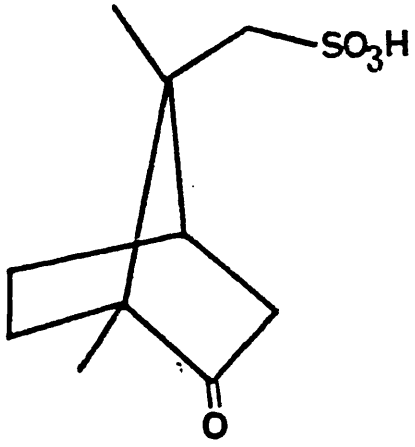
Alkylsulphates



12-dien-Zn(II)



D,L-carboethoxyheptyltrimethylammonium methanesulphonate



D-camphorsulphonic acid

2.1.5 HPLC packing materials

The following HPLC packing materials were used: ODS*Hypersil SAS Hypersil (Shandon Southern, Runcorn, U.K.), Spherisorb S5 ODS, Spherisorb Hexyl (Phase Separations, Queensferry, U.K.), Partisil 10 ODS, Partisil 10 ODS 2 and Partisil 5 (Whatman Ltd., Maidstone, U.K.).

* ODS is a standard abbreviation for octadecylsilica.

Table 2.3 Compound codes, purities and sources of the benzoic acids

Benzoic acid	Code	Purity[1]	
4-amino	4-ABA	>99%	BDH, Poole, U.K.
3-amino	3-ABA	>99%	Fisons, Loughborough, U.K.
2-amino	2-ABA	>98%	Aldrich, Gillingham, U.K.
4-hydroxy	4-HBA	>99%	BDH, Poole, U.K.
3-hydroxy	3-HBA	>95%	Aldrich, Gillingham, U.K.
2-hydroxy	2-HBA	AnalaR	BDH, Poole, U.K.
4-methyl	4-MBA	>99%	BDH, Poole, U.K.
3-methyl	3-MBA	>99%	BDH, Poole, U.K.
2-methyl	2-MBA	>99%	BDH, Poole, U.K.
4-nitro	4-NBA	>99%	Aldrich, Gillingham, U.K.
3-nitro	3-NBA	>99%	Aldrich, Gillingham, U.K.
2-nitro	2-NBA	>99%	Fisons, Loughborough, U.K.
4-chloro	4-CBA	>99%	BDH, Poole, U.K.
3-chloro	3-CBA	>99%	BDH, Poole, U.K.
2-chloro	2-CBA	>99%	BDH, Poole, U.K.
Phenylacetic	PAA	>99%	BDH, Poole, U.K.
Cinnamic	CA	>99%	BDH, Poole, U.K.
Benzoic	BA	AnalaR	BDH, Poole, U.K.
4-phthalic	4-PA	>99%	BDH, Poole, U.K.
3-phthalic	3-PA	>99%	BDH, Poole, U.K.
2-phthalic	2-PA	>99%	BDH, Poole, U.K.

1. Manufacturer's specification

Table 2.4 Compound codes of the tryptophan metabolites (supplied by Sigma and of "Sigma Grade")

Tryptophan Metabolite	Compound Code
Tryptophan	Trp
5-hydroxy-tryptophan	5-HTP
5-hydroxy-tryptamine	5-HT
5-hydroxy-indole-3-acetic acid	5-HIAA
Kynurenine	Kyr
3-hydroxy-anthranilic acid	3-HAA

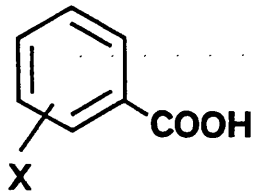
Table 2.5 Compound codes of the histidine metabolites, (supplied by Sigma and of "Sigma Grade")

Histidine Metabolite	Compound Code
Histidine	His
Imidazole-4-acetic acid	IAA
Urocanic acid	UA

Table 2.6 Compound codes of phenylalanine metabolites (supplied by Sigma and of "Sigma Grade")

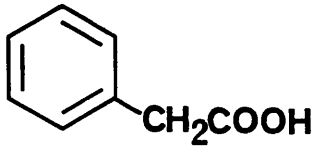
Phenylalanine Metabolite	Compound Code
Phenylalanine	Phe
3,4-dihydroxyphenylalanine	DOPA
Tyrosine	Tyr
4-chlorophenylalanine	CPA
4-hydroxyphenylpyruvic acid	HPPA
4-hydroxy-3-methoxy-mandelic acid	HMMA
3,4-dihydroxy-mandelic acid	HMA
Homogentisic acid	HG

Fig. 2.2.a The structure of the substituted benzoic acids, 1,3,5-s-triazines and 8-azapurin-6-ones

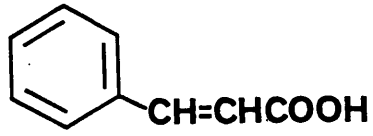


Benzoic acids

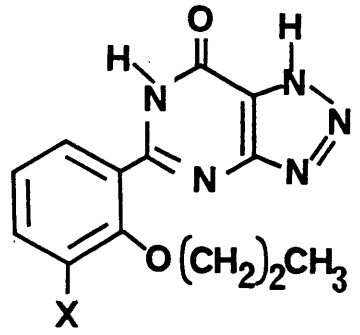
X; 4-,3-,2-, NH_2
4-,3-,2- OH
4-,3-,2- NO_2
4-,3-,2- CH_3
4-,3-,2- Cl



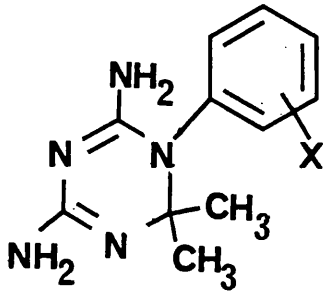
Phenylacetic acid



Cinnamic acid

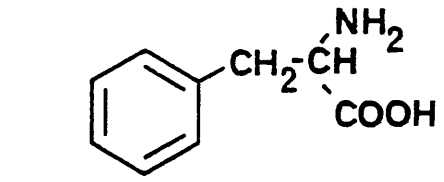


8-azapurin-6-ones
X; H, SO_2NH_2, NH_2, CN,
$SO_2C_3H_7$, SH, $CO_2C_2H_5$
OC_4H_9, $C(CH_3)_3$

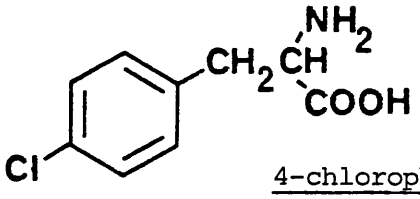


1,3,5-s-triazines
3-X; n-C_6H_{13}, $C(CH_3)_3$, $N(CH_3)_2$,
SCH_3, Br, CN, SO_2CH_3,
OH, $(CH_2)_4$-phenyl,
$(CH_2)_4$-phenyl-4-OCH_3,
O-n-C_6H_{13}, O-n-C_9H_{19}
4-X; n-C_6H_{13}, SCH_3, CF_3, Br,
$NHCOCH_3$, CN, OH, SO_2CH_3
SO_2NH_2, $C(CH_3)_3$.

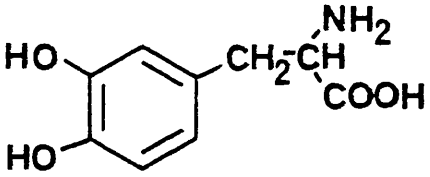
Fig. 2.2.b The structures of the phenylalanine metabolites



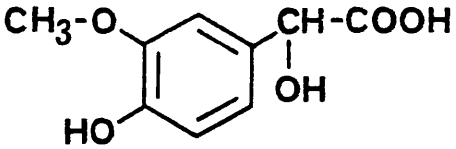
Phenylalanine



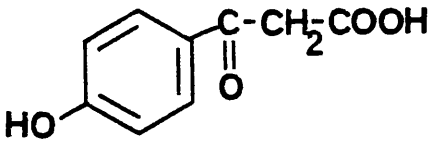
4-chlorophenylalanine



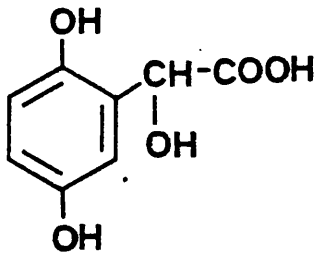
3,4-dihydroxyphenylalanine (DOPA)



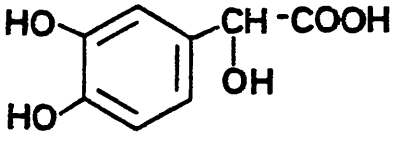
4-hydroxy-3-methoxy mandelic acid



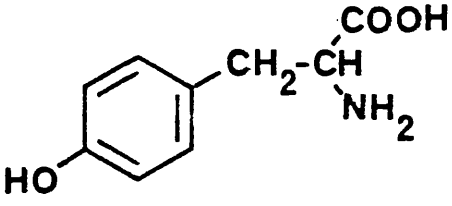
4-hydroxyphenylpyruvic acid



Homogentisic acid

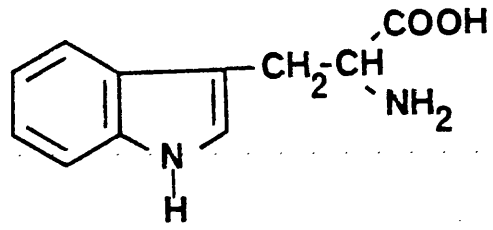


3,4-dihydroxymandelic acid



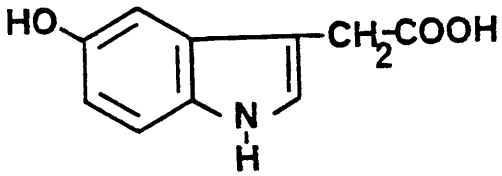
Tyrosine

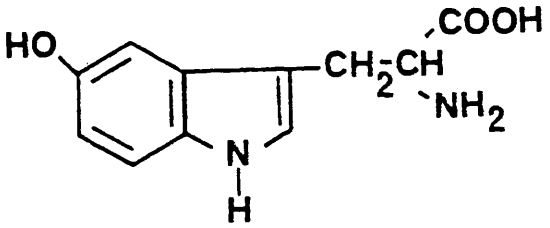
Fig. 2.2.c The structures of the tryptophan metabolites



Tryptophan

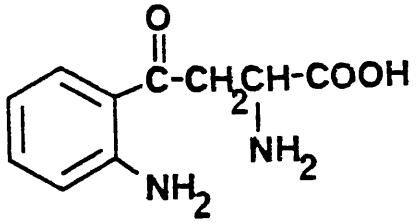
5-hydroxyindole-3 -acetic acid

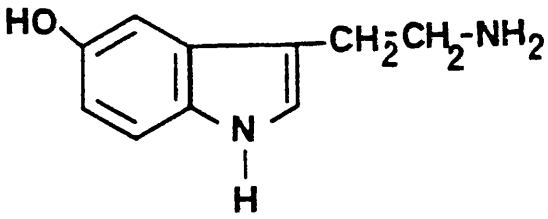




5-hydroxytryptophan

Kynurenine





5-hydroxytryptamine

3-hydroxyanthranilic acid

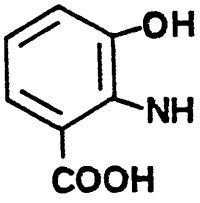
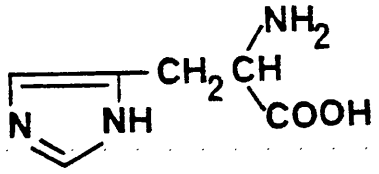
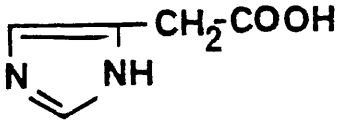


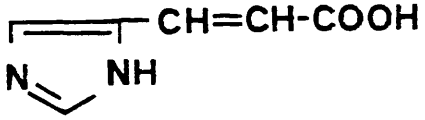
Fig. 2.2.d The structures of the histidine metabolites



Histidine



Imidazole-4-acetic acid



Urocanic acid

Fig. 2.2.e The structure of sodium cromoglycate

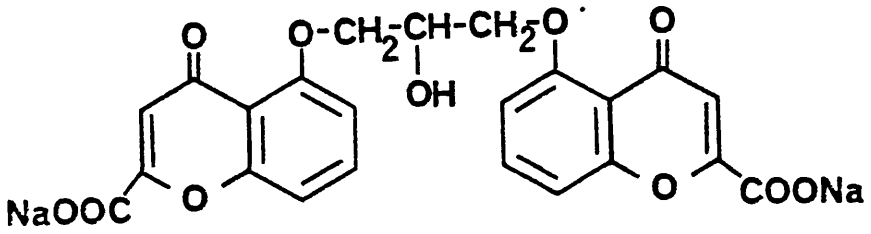
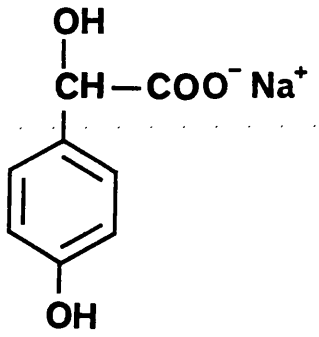
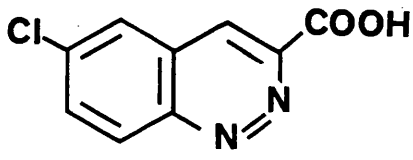


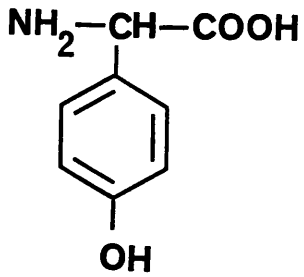
Fig. 2.2.f The structures of sodium 4-hydroxymandelate, 6-chlorocinnoline-3-carboxylic acid, 4-hydroxyphenylglycine and 2-hydroxyphenylglycine.



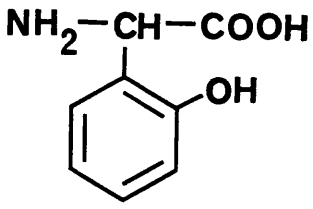
sodium 4-hydroxymandelate (SHM)



6-chlorocinnoline-3-carboxylic acid (CCA)

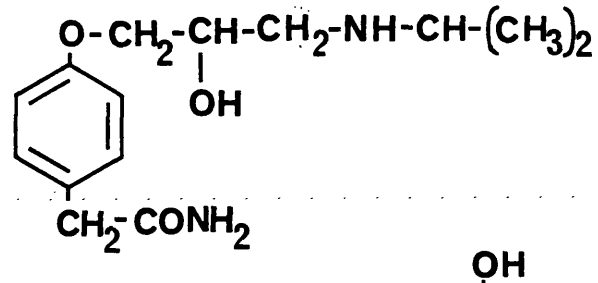


4-hydroxyphenylglycine (4-HPG)

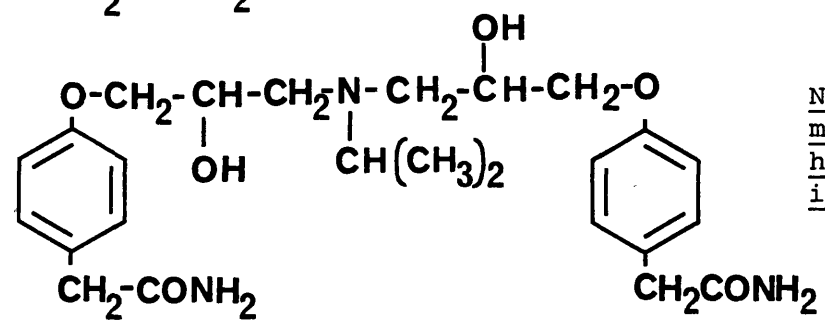


2-hydroxyphenylglycine (2-HPG)

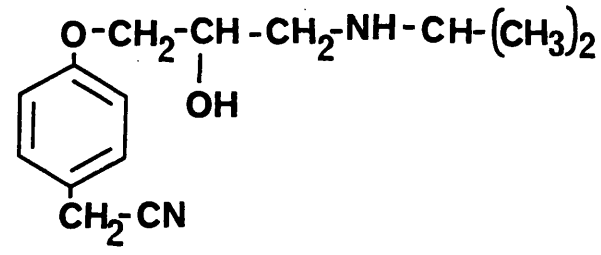
Fig. 2.2.g The structures of atenolol (I) and five related compounds (series 1)

I 

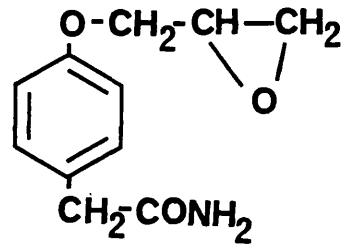
Atenolol. (4-(2'-hydroxy-3'-isopropylamino propoxy)-phenylacetamide)

II 

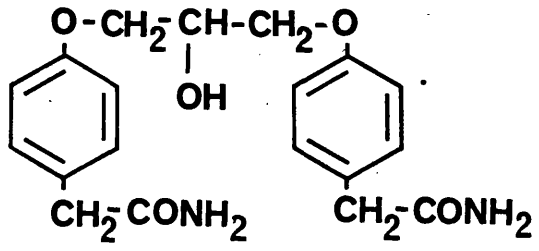
N,N,di(3-(4-carbamoyl-methyl) phenoxy-2-hydroxypropyl) isopropylamine

III 

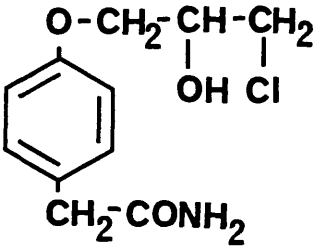
4-(2'-hydroxy-3'-isopropyl aminopropoxy)-benzylcyanide

IV 

1-(4'-carbamoylmethylphenoxy)2:3-epoxypropane

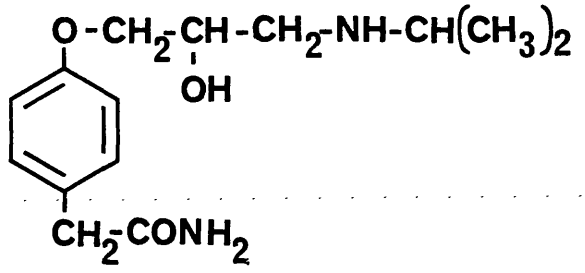
V 

1,3-bis(4-carbamoylmethylphenoxy) -propan-2-ol

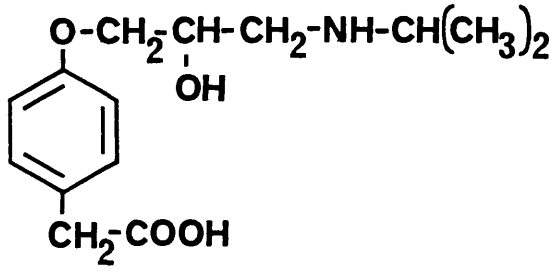
VI 

1-(4-carbamoylmethylphenoxy)-3-chloropropan-2-ol

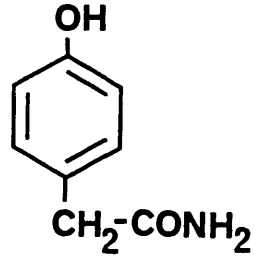
Fig. 2.2.h Structures of atenolol and related compounds (series 2)

I 

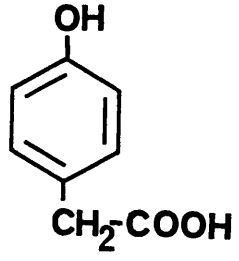
Atenolol

VII 

4-(2'-hydroxy-3'-isopropyl-aminopropoxy)-phenyl-acetic acid

VIII 

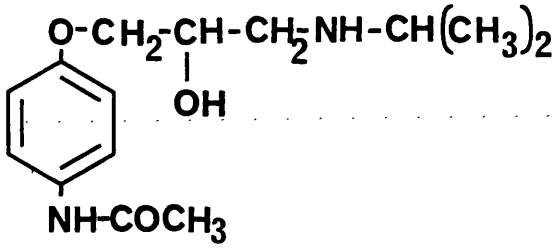
4-hydroxyphenylacetamide

IX 

4-hydroxyphenylacetic acid

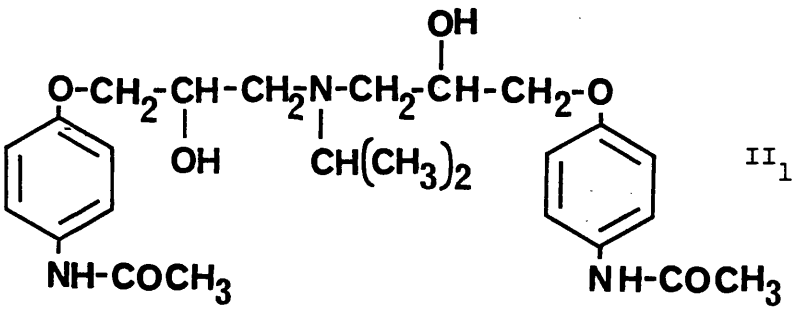
Fig. 2.2. i The structures of practolol and two related compounds

I_1



Practolol (4-(2-hydroxy-3-isopropylaminopropoxy)-acetanilide

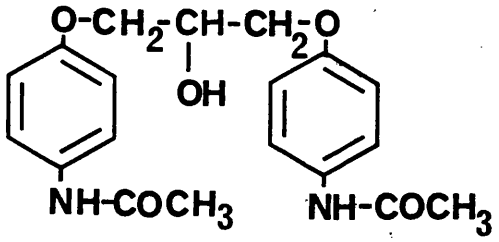
II_1



II_1

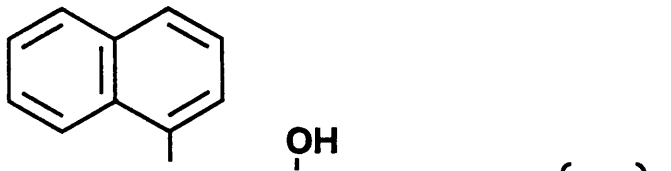
N,N-bis 3-(4-acetamidophenoxy)-2-hydroxypropyl isopropylamine

V_I

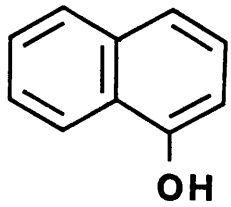


1,3-bis(4-acetamidophenoxy)-propan-2-ol

Fig. 2.2.j Structures of propranolol and 1-naphthol

XIV 

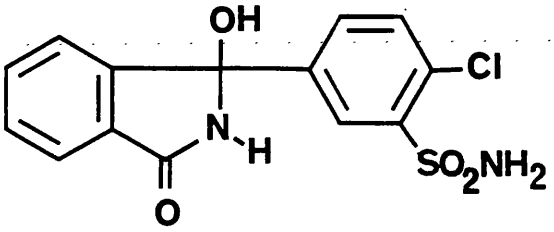
Propranolol (1-isopropylamino-3-(1-naphthyloxy)-propan-2-ol)



1-naphthol

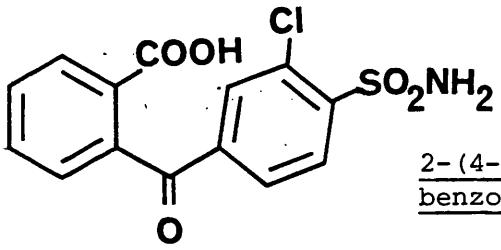
Fig. 2.2.k The structures of the diuretics chlorthalidone and bendrofluazide and their respective degradation products.

X



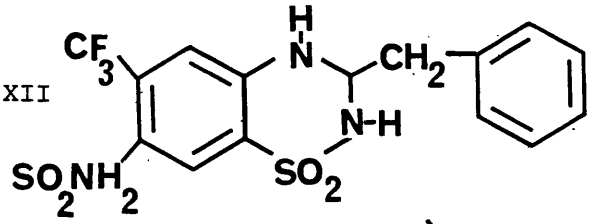
Chlorthalidone (3-(4-chloro-3-sulphamoylphenyl)-3-hydroxyisoindolin-1-one

XI



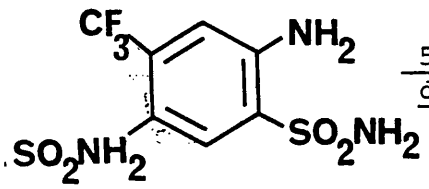
2-(4-chloro-3-sulphamoyl benzoyl)-benzoic acid

XII



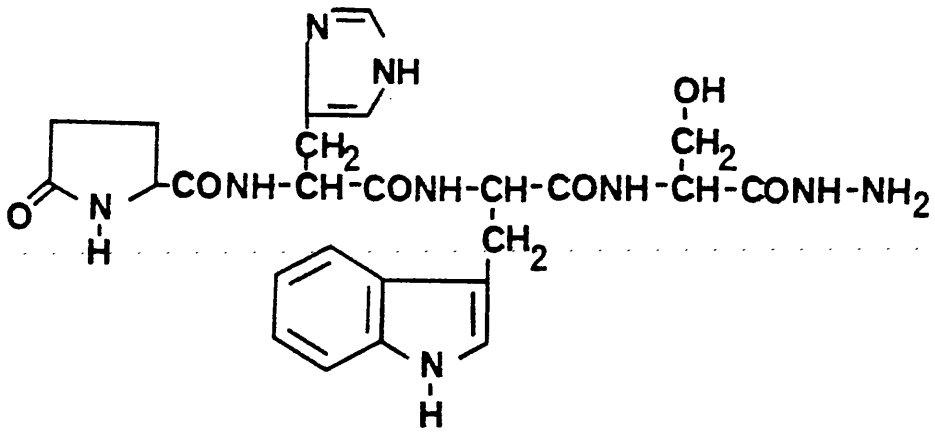
Bendrofluazide (3-benzyl-3,4-dihydro-6-trifluoromethylbenzo-1,2,4-thiadiazine-7-sulphonamide 1,1-dioxide)

XIII

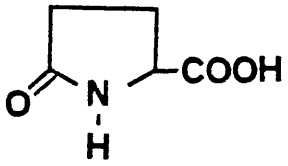


5-trifluoromethyl-2,4-disulphonamidoaniline

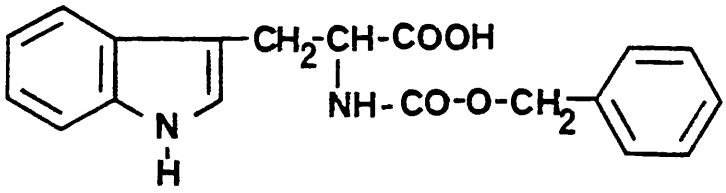
Fig. 2.2.1 Structures of the Tetrapeptide, Glu.His.Trp.Ser.NH.NH_2 and its'protected ' amino acid sub-units.



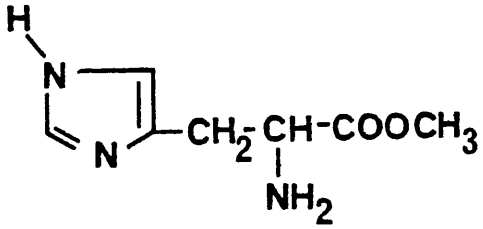
Glu.His.Trp.Ser.NH.NH_2



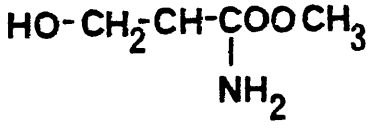
L-pyroglutamic acid (L-Glu)



Carbobenzoxy-L-tryptophan. (CBZ-L-Trp)

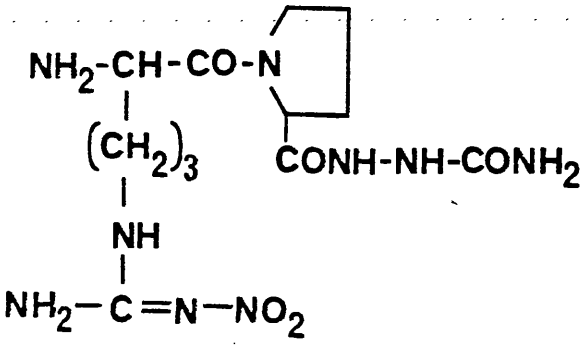


L-histidine-methyl ester. (Me-L-His).

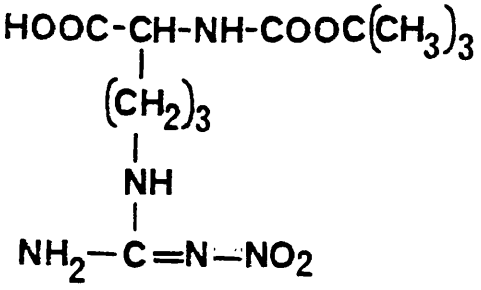


L-serine-methyl ester. (Me-L-Ser).

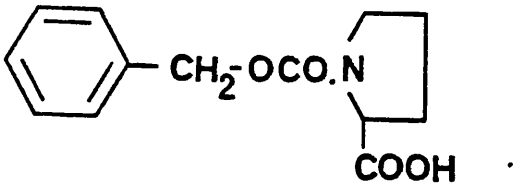
Fig. 2.2.m Structures of the tripeptide, nitro-L-arginyl-L-prolyl-azaglycine amide and its 'protected' amino acid subunits.



NO_2.arg.pro.gly.NH.NH.Co.NH_2

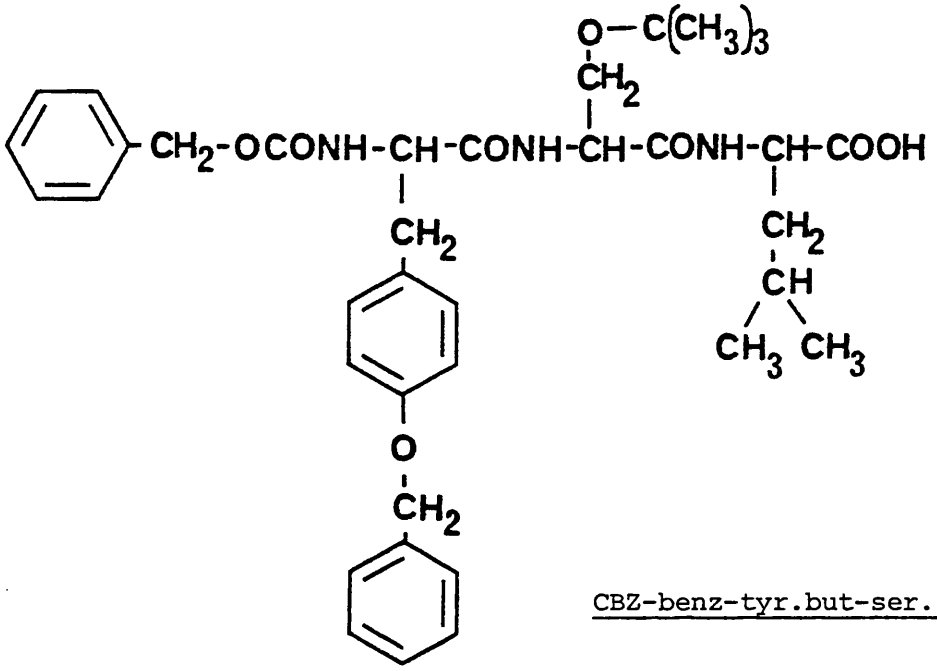


N-t-butyloxycarbonyl-N-nitro-L-arginine (N-BOC-NO_2.Arg)

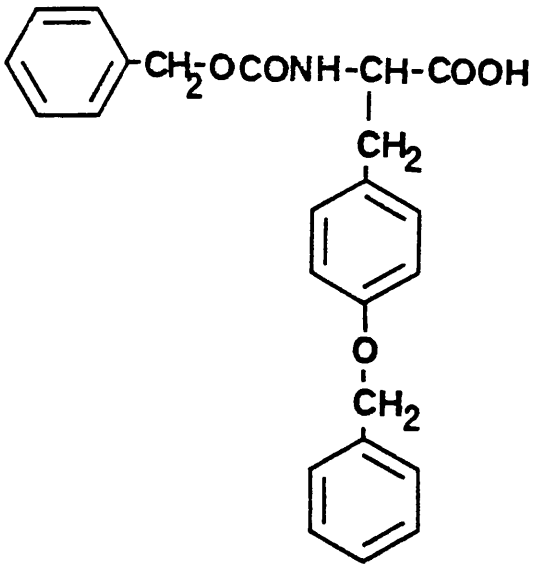


N-carbobenzoxy-L-proline, (CBZ-Pro)

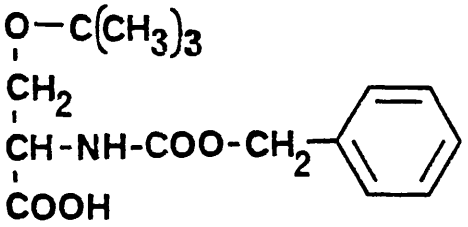
Fig. 2.2.n Structures of the tripeptide, N-carbobenzoxy-O-benzyl-L-tyrosyl-O-t-butyl-D-seryl-L-leucine, and its 'protected' amino acid sub-units.



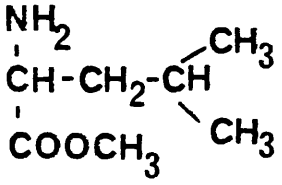
CBZ-benz-tyr.but-ser.leu



N-carbobenzoxy-O-benzyl-tyrosine (CBZ-benz-Tyr)



N-carbobenzoxy-O-t-butyl-D-serine (CBZ-but-Ser)



L-leucine methyl ester (Me-Leu)

2.2 Equipment

2.2.1 Conductivity apparatus

The conductivity apparatus consisted of a Mullard dip type electrolytic conductivity cell (Sproule, London, U.K.) placed in a 100 ml. straight sided cylinder and connected to a Phillips conductivity meter (type PW 9501). The contents of the measuring cylinder were agitated by means of a magnetic stirrer and the temperature measured by means of a mercury thermometer.

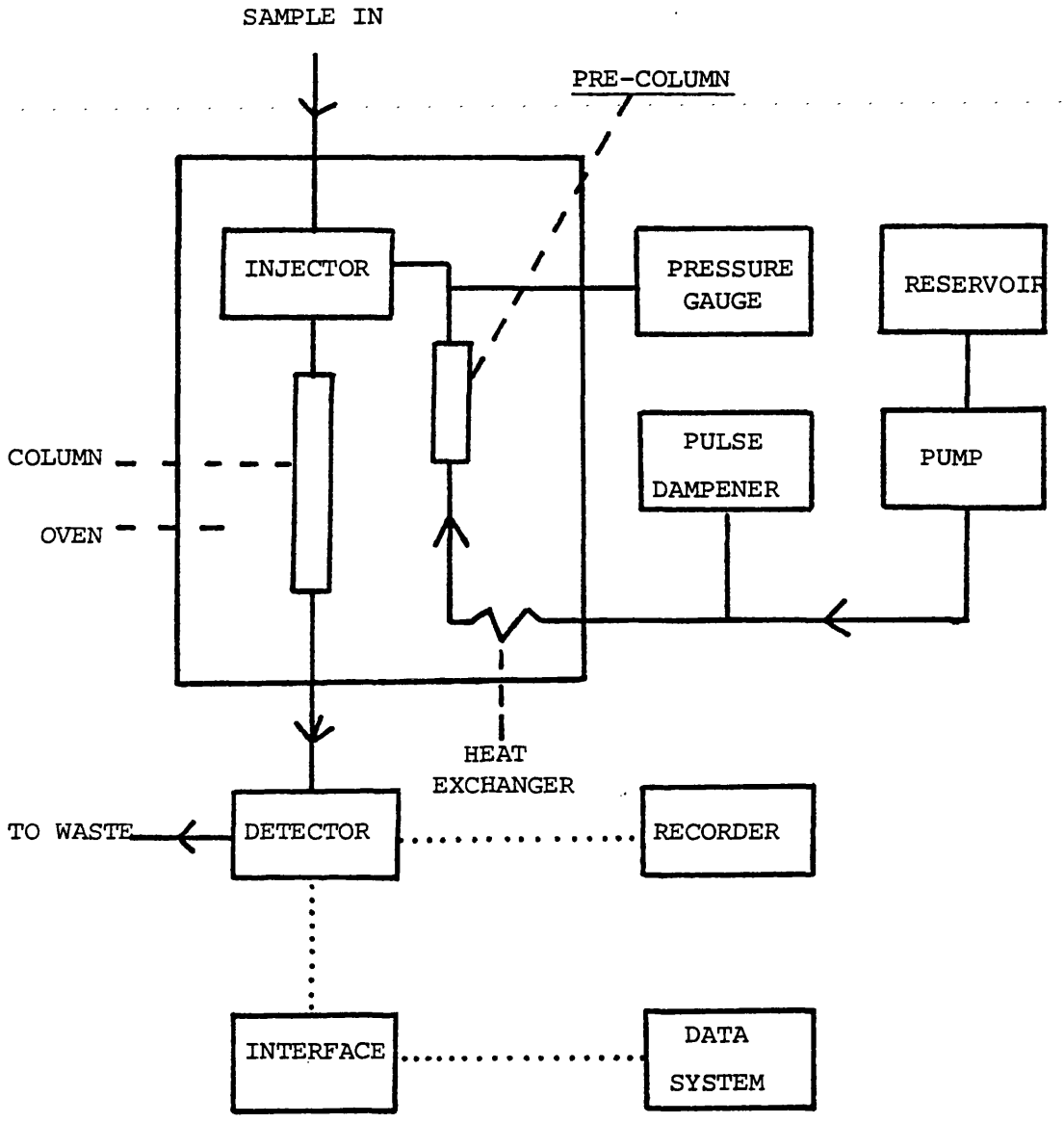
2.2.2 Liquid chromatographs

Several liquid chromatographs were assembled from commercially available components. Figure 2.3 shows a generalised scheme of the chromatographs used.

The mobile phase was pumped to the column by means of a liquid pump, and the column eluent monitored by passage through the flow cell of the detector. The output of the detector was recorded on a potentiometric chart recorder (Bryans 28000, Bryans Southern Instruments Ltd., Mitchen, U.K.). The signal from the detector was also monitored by either a Perkin Elmer Sigma 10 data station (Perkin-Elmer, Slough, U.K.) or a Hewlett Packard 3350 laboratory computer.

Sample injections were made either by means of a 10 µl syringe (Hamilton, type 75N, Phase Separations, Queensberry, U.K.) through a septum injector (HETP, Macclesfield, U.K.) or means of a loop valve (Rheodyne 905-42 or Specac PN 30500, Jones Chromatography, Llanbradach, U.K.). On a number of occasions injections were made using a Varian 8050 autoinjector (Varian Association, Walton-on-Thames, U.K.).

Fig. 2.3 Generalised scheme of the liquid chromatograph



The column eluent was monitored either by UV spectrometry using a Cecil CE 212 or a Cecil CE 2102 variable wavelength spectrometer (Cecil Instruments, Cambridge, U.K.) fitted with 8 µl flow cells, or by polarography using an EDT electrochemical detector (type LCA 10 EDT Research, London, U.K.) fitted with an 4 µl flow cell and a glassy carbon electrode.

Both constant flow rate (Constametric I Jones Chromatography, or LDC Liquid delivery System LDC, Stone, U.K.) and constant pressure (mini-Haskel type 28, Olin-Energy, Sunderland, U.K.) pumps were used. When using the constant pressure pump it was necessary to incorporate a pulse dampener.

The chromatography columns consisted of 5 mm internal diameter (¼" external diameter) stainless steel tubes of various lengths (ex. HETP). The column end fitting consisted of a low dead-volume Swagelok connection with a 1/16" outlet which was connected to the detector by microbore (internal diameter 0.15 mm) Teflon tubing. Low dead volume 1/16" stainless steel tubing (internal diameter 0.15 mm) was used to connect the injection valve to the top of the column. All the other connections were made using stainless steel tubing and standard Swagelok fittings (HETP).

Whenever practical the temperature of the column and injector was thermostatically controlled by immersion in a modified Perkin Elmer F11 oven or in a heated water bath (Gallenkamp, type 400-010). The mobile phase was pre-heated to the column temperature by passage through a heat exchanger placed in the oven or water bath.

2.2.3 HPLC column packing apparatus

The column packing apparatus (see Fig. 2.4) consisted of a 3/8" external diameter stainless steel tube, 50 cm. in length, which served as the slurry reservoir. One end of the slurry reservoir was connected to a mini-Haskell pump and the other end was attached to a 1/4" external diameter stainless steel guard column. Prior to packing the column, the guard column was removed and attached to the empty column tube.

2.3 Procedures

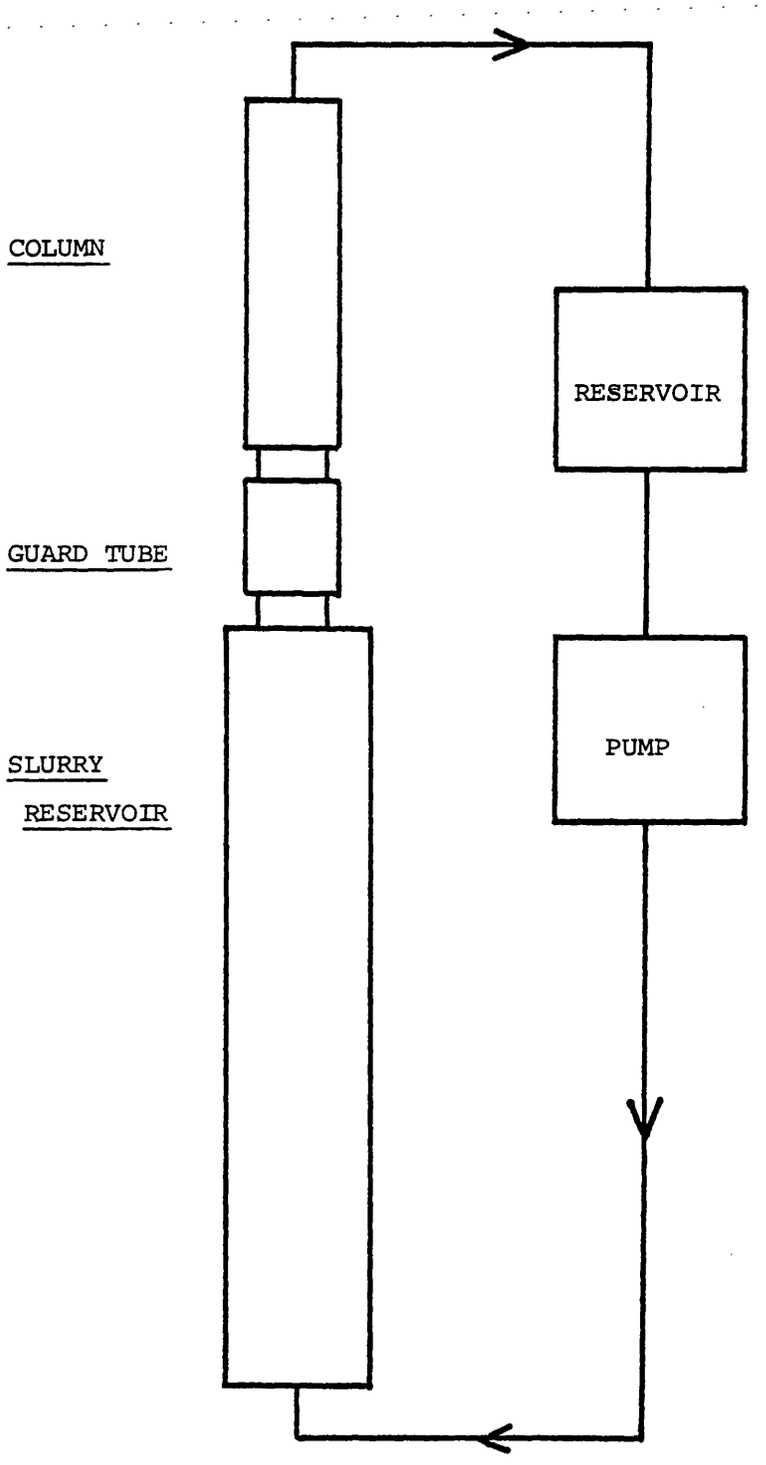
2.3.1 Critical micelle concentration determinations

The critical micelle concentration (CMC) of C_{14}BDAC was determined using the conductivity apparatus described in Section 2.2.1. Deionised water (50 mls) was pipetted into a 100 ml. cylinder and stirred. After one minute the stirrer was stopped and the conductivity measured. Aliquots (1 ml) of a 2.5 x 10^{-2} mol. dm^{-3} C_{14}BDAC solution in deionised water were pipetted into the cylinder. The contents were stirred for one minute and the conductivity of the solution measured as before.

The conductivity of the C_{14}BDAC solution was plotted against concentration and the CMC determined from the intersection in slopes.

To determine the effect of methanol on the CMC of C_{14}BDAC, the experiment was repeated using methanolic solutions of C_{14}BDAC containing 10, 20, 40 and 50% v/v methanol. Results are given in Tables 2.7a, 2.7d and shown graphically by Fig. 2.5.

Fig. 2.4 Column packing apparatus



2.3.2 Column packing

All the columns were packed using the upward slurry packing technique described by Bristow *et al.* (27). Initially, the slurrying solvent used was methanol, however it was found later that columns could be packed more satisfactorily using a slurry medium of 10% v/v methanol in carbon tetrachloride.

For a 100 mm. x 5 mm (id) column, 1.3 gm. of packing material was weighed into a 50 ml volumetric flask and dispersed in about 15 mls of solvent by immersion in an ultrasonic bath. The pump was then charged to full pressure (450 Bar) and the slurry poured into the slurry reservoir. The guard column and the column blank were then attached to the slurry reservoir. The slurry was then pumped through the column at maximum pressure for at least 30 minutes. After switching off the flow, the column was not removed until the pressure in the system had fallen to zero.

After removing the column from the packing apparatus the top 5 mm of packing material were removed and a 2 μm stainless steel mesh inserted . When syringe injection was to be employed, Ballotini beads (diameter 70 - 150 μm) were poured onto the top of the mesh to a depth of 3 mm. The remaining gap above the beads was filled with a porous Teflon frit.Syringe injections were made by pushing the tip of the needle through the Teflon frit into the beads. Ballotini beads were not used when making valve injections since they tended to block the column inlet tube. When using a loop valve, the column inlet tube was carefully positioned above the stainless steel mesh and the remaining gap filled with a Teflon collar.

Table 2.7a The relationship between conductivity and the concentration of C_{14}BDAC in deionised water at 20 °C.

C_{14}BDAC (mol.dm^{-3}) x 10^3	Conductivity (scale units)
0.00	13
0.49	50
0.97	83
1.42	121
1.86	153
2.29	175
2.69	191
3.09	205
3.47	224
3.83	238
4.19	251
4.53	264
4.87	276
5.19	287
5.49	299
5.80	308

Table 2.7b The relationship between conductivity and the concentration of C_{14} BDAC in 10% v/v methanol in water at 20°C

C_{14}BDAC (mol.dm^{-3}) x 10^{3}	Conductivity (scale units)
0.00	0
0.49	36
0.96	72
1.41	103
1.85	132
2.27	156
2.67	182
3.06	200
3.44	216
3.80	230
4.16	243
4.50	255
4.82	267

Table 2.7c The relationship between conductivity and the concentration of C_{14}BDAC in 20% methanol in water at 20°C

C_{14}BDAC (mol.dm^{-3}) x 10^3	Conductivity (scale units)
0.00	44
0.39	68
0.77	98
1.23	121
1.48	143
1.81	163
2.14	182
2.45	199
2.75	215
3.04	231
3.32	236
3.60	249

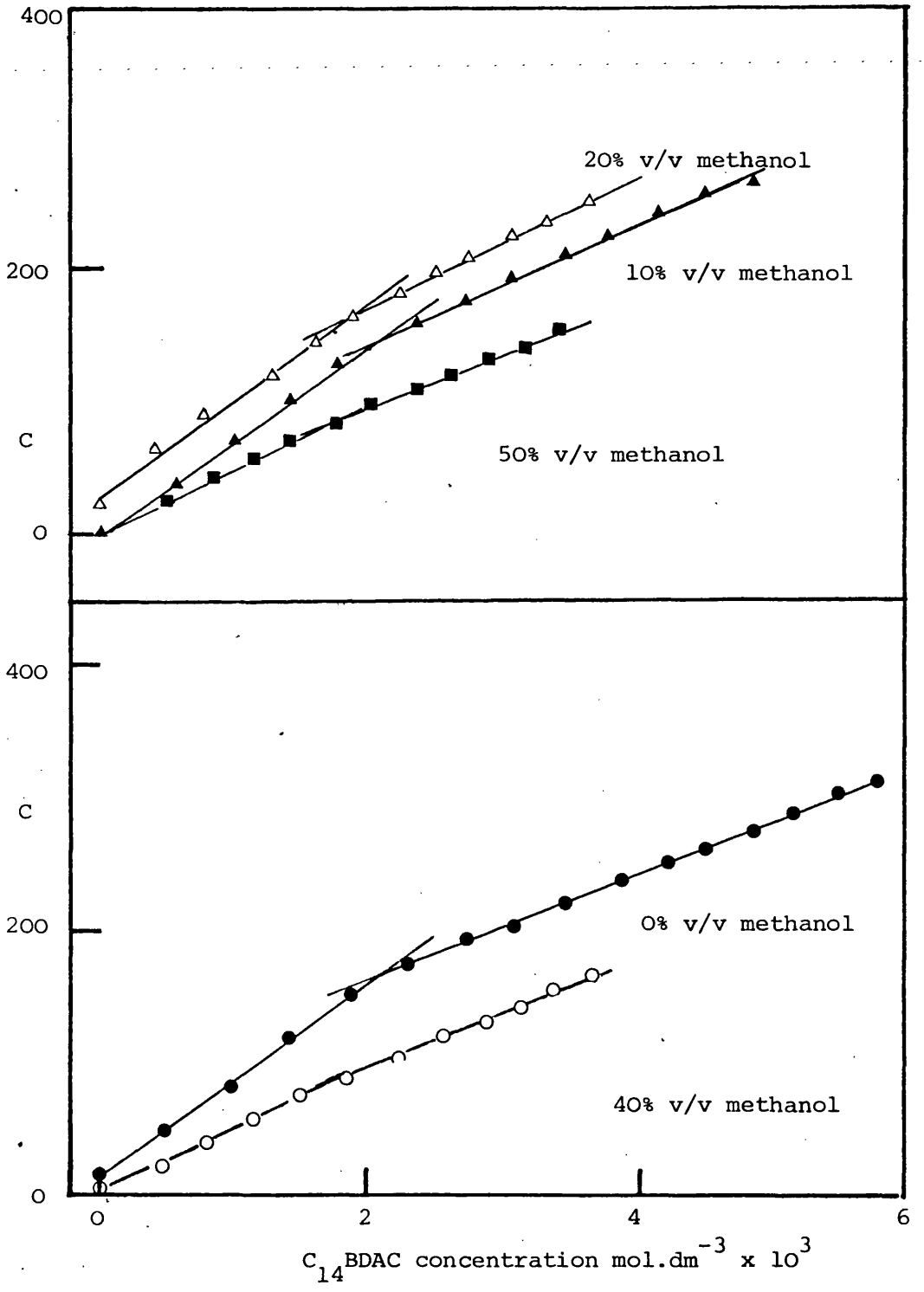
Table 2.7d The relationship between conductivity and the concentration of C_{14}BDAC in 40% v/v methanol in water at 20°C

C_{14}BDAC (mol.dm^{-3}) x 10^3	Conductivity (scale units)
0.00	0
0.40	23
0.78	42
1.16	59
1.51	76
1.85	88
2.18	101
2.50	120
2.81	134
3.11	143
3.33	160
3.68	172

Table 2.7e The relationship between conductivity and the concentration of C_{14}BDAC in 50% v/v methanol in water at 20°C

C_{14}BDAC (mol.dm^{-3}) x 10^3	Conductivity (scale units)
0.00	2
0.38	23
0.74	38
1.09	55
1.42	70
1.74	80
2.06	98
2.36	111
2.65	118
2.93	135
3.20	146
3.46	158

Fig. 2.5. Conductiometric critical micelle concentration (CMC) determination of C_{14}BDAC at different methanol concentrations, showing conductivity (C) (in arbitrary scale units) *versus* C_{14}BDAC concentration, 20°C.



2.3.3 Column Testing

The quality of the column packing was tested according to the methods described by Bristow (14) and Bristow and Knox (204). For reversed phase columns, the test solution was acetone (0.40% v/v), anisole (0.03% v/v) and phenetole (0.07% v/v) in methanol:water (7:3). The mobile phase composition for testing reversed phase columns depended upon the carbon loading, the stationary phase and was adjusted to similar retentions for the test solutes. For silica columns, a test mixture of toluene (0.05% v/v), phenanthrene (0.01% w/v) and nitrobenzene (0.01% v/v) in pentane with a mobile phase of 1% v/v acetonitrile in n-hexane was used.

The data obtained from the column testing was analysed using a modified computer programme, described by Bristow *et al*. (27). Columns giving a reduced plate height of more than 4.5 or abnormal porosity values were rejected.

Five reversed phase columns were also tested to determine the extent of their residual hydroxyl groups by examining the retention of nitrobenzene using a mobile phase of sodium dried n-hexane (Table 2.10)

2.3.4 Aqueous mobile phase preparation

All the aqueous mobile phases were prepared by pipetting the appropriate amounts of buffer solution, pairing-ion solution and organic solvent into a grade B volumetric flask and adjusting to volume with distilled water. The final volume adjustment was made after cooling the mobile phase to room temperature.

Table 2.8 Summary of the column lengths (L), internal diameters (d_c) nominal particle diameters (d_p), effective spherical diameters (d_{sp}) and porosities (e) of the HPLC columns

Column Code	Stationary Phase	L (mm)	d_c (mm)	d_p (µm)	d_{sp} (µm)	e
A	Spherisorb S5 ODS	95	5	5	4.86	0.74
B	Spherisorb S5 ODS	45	5	5	5.09	0.78
C	Partisil 10 ODS	45	5	10	6.74	0.67
D	Spherisorb Hexyl	45	5	5	5.36	0.67
E	Partisil 10 ODS 2	45	5	10	7.33	0.60
F	ODS Hypersil	45	5	5	4.93	0.73
G	ODS Hypersil	95	5	5	4.80	0.76
H	ODS Hypersil	195	5	5	5.24	0.74
I	SAS Hypersil	95	5	5	4.86	0.74
J	Spherisorb S5 ODS	195	5	5	4.86	0.74

Table 2.9 Capacity ratios (κ), and reduced plate heights (h) of the test solutes acetone, anisole and phenetole for the columns described in Table 2.8. Mobile phase: methanol:water, injection volume: 10 μm., flow rate: 2.0 $ml.min^{-1}$ (33 $\mu l.s^{-1}$), temperature 30°C.

Column	Mobile Phase ($MeOH:H_2O$)	Acetone		Anisole		Phenetole	
		κ	h	κ	h	κ	h
A	50:50	0.19	3.96	2.33	3.86	4.23	3.77
B	50:50	0.20	4.22	2.32	4.30	4.20	4.32
C	30:70	0.22	3.43	3.33	3.18	6.00	3.30
D	40:60	0.27	4.41	3.11	4.28	5.28	4.45
E	70:30	0.25	3.77	2.94	3.61	4.44	3.86
F	60:40	0.16	3.70	2.29	3.91	4.06	3.87
G	60:40	0.16	3.51	2.37	3.16	4.23	3.32
H	60:40	0.12	2.47	2.33	2.55	4.20	2.40
I	50:50	0.29	3.81	2.10	3.58	3.52	4.13
J	50:50	0.20	2.50	2.30	2.89	3.98	2.77

Table 2.10 Summary of the surface properties of five reversed phase packing materials, showing the surface area, carbon loadings, bonded alkyl chain lengths and the capacity ratios of nitrobenzene eluted with a mobile phase of dry n-hexane.

Stationary Phase	Column Code	Surface Area[a] ($m^2.g^{-1}$)	Carbon Loading[b] (% w/w)	Chain Length	κ of Nitrobenzene
ODS Hypersil	F	200	9.9	18	0.19
Spherisorb S5 ODS	B	220	5.9	18	3.10
Partisil 10 ODS 2	E	400	15.0	18[c]	3.60
Partisil 10 ODS	C	400	3.9	18	5.80
Spherisorb hexyl	D	220	4.6	6	0.56
Partisil 5	K	400	0	-	6.80

a. Manufacturers specification

b. By elemental analysis

c. The alkyl chains of this material also have some cross linkages, and may be considered to be partially polymeric in nature.

The buffer stock solutions were stored for not more than two weeks at 4°C. All glassware in contact with surfactant solutions was cleaned with chromic acid to remove prior adsorbed surfactant. To minimise surfactant adsorption effects the surfactant stock solutions and mobile phases were prepared in containers which had been aged for at least 24 hours with surfactant.

After the final volume adjustment, the pH of the mobile phase was determined using a pH meter (Pye-Unicam, Cambridge, U.K.) and slight adjustments were made, if necessary, by dropwise addition of N10 NaOH or H_3PO_4. Except when specifically investigating the effect of pH, the pH of the mobile phases was such that the solutes were in their fully ionised state.

Before use the mobile phase was degassed by either refluxing for 10 minutes or purging with helium for five minutes. The latter method proved to be the most convenient and was the most commonly used.

2.3.5 Reversed phase column adsorption of pairing-ions

A Spherisorb S5 ODS (A) column was used to determine the effect of ABDAC concentration and alkyl chain length on the adsorption of pairing-ions onto the surface of the stationary phase.

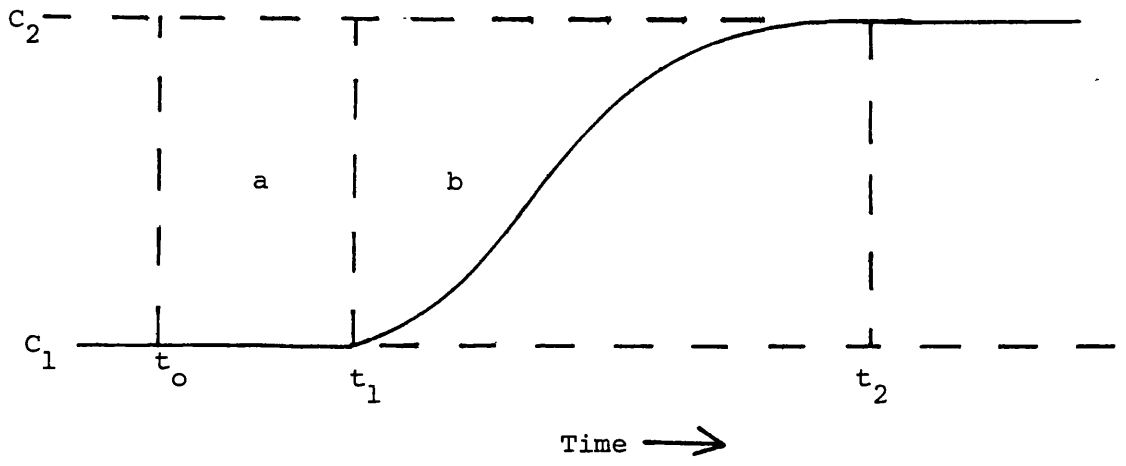
For this study, the injection valve was removed and the pump connected directly to the column. Three homologues (8, 10 and 13) at 5 or 6 concentrations over the range 0 to 10^{-3} mol. dm^{-3} in methanol:water (1:1)were studied. Each ABDAC solution was pumped through at a constant flow rate until a rise in baseline attributed

to column equilibration was observed (see Fig. 2.6). Knowing that the ABDACs obey Beer's law over the concentration range studied then the column adsorption, AD, was calculated according to the following relationship:

$$AD = (C_2-C_1)F_c.(t_1-t_o)(w_a+w_b)w_a^{-1} \quad (99)$$

where C_1 and C_2 are the initial and final ABDAC concentrations, F_c is the flow rate and w_a and w_b are the weights of chart paper of areas a and b, respectively (see Fig. 2.6).

Fig. 2.6 Change in recorder baseline (solid line) for an alteration in ABDAC concentration (C_1 to C_2).



After each change in ABDAC concentration the dead volume was purged. After each change in ABDAC homologue, the adsorbed surfactant was removed from the column by either passing through a 5×10^{-4} mol. dm^{-3} sodium cromoglycate solution for 30 minutes followed by methanol: water (1:1) for 10 minutes or by flushing with methanol for 40 minutes.

Table 2.11 The adsorption, AD,of ABDAC from mobile phases containing different concentrations and alkyl chain lengths of ABDAC pairing-ions. Stationary phase: Spherisorb S5 ODS (Column A). Mobile phase: methanol:water (1:1) + ABDAC. Temperature 20°C

C_8BDAC		C_{11}BDAC		C_{13}BDAC	
Concentration $mol.dm^{-3} \times 10^4$	AD $mol.kg^{-1} \times 10^3$	Concentration $mol.dm^{-3} \times 10^4$	AD $mol.kg^{-1} \times 10^3$	Concentration $mol.dm^{-3} \times 10^4$	AD $mol.kg^{-1} \times 10^3$
0.51	0.52	0.58	3.01	1.03	3.97
1.03	1.22	2.23	7.12	2.06	8.30
2.05	2.73	5.57	12.2	5.14	13.3
5.13	6.38	7.24	14.0	10.3	30.2
10.3	14.2	11.1	22.9		

2.3.6 Determination of chromatographic parameters

a. Capacity ratio (κ).

The capacity ratios were determined at least in duplicate and were obtained from retention data according to Eq. 8. Water or methanol was used for the determination of t_o in reversed phase systems and n-pentane was used in straight phase systems. The retention times were measured using either one of the data systems described previously or an electronic stopwatch.

Great care was taken to ensure that the measurement of retention times was made after equilibration of the column and mobile phase. Column equilibration was found to occur rapidly when changing the organic solvent composition but much more slowly when changing the surfactant pairing-ion concentration.

b. Column efficiency.

The calculations of column efficiency were made according to Eq. 2. A fast chart speed (1 mm. s^{-1}) was used to facilitate accurate measurement of $w_{\frac{1}{2}}$ which was made with an eyepiece micrometer.

c. Flow rate (F_c).

The mobile phase flow rate was obtained by placing the detector outlet tube in a 10 ml. standard flask and noting the time at which the level of the liquid passed the graduation. Unless specified a flow rate of 2.0 ml. min^{-1} was used throughout.

2.3.7 Solute preparation.

The solute solutions were generally prepared using the appropriate mobile phase, the concentrations depending upon the detector response,

but were usually less than 1.0 mgm.ml^{-1}. (The concentration of organic solvent was increased when solutions of very hydrophobic solutes were prepared).

2.3.8 Analytical procedures.

a. Analytical and internal standards.

Biological samples and pharmaceutical formulations were assayed after the appropriate pre-treatment by comparison with analytical standards of known concentration. The assays were performed both with and without internal standard. Stock solutions of internal standards, which did not interfere with the separation of analytes, were prepared. To determine the reproducibility of injection, a single standard solution was injected ten times and the standard deviations of the peak heights (or peak areas) and peak height (or peak area) ratios determined. When using accurate injection valves satisfactory relative standard deviations (i.e. <2%) were obtained for peak height (or peak area) and in such cases assays were made without the use of internal standards.

b. Linearity of response.

Calibration curves were prepared by determining the peak heights (or peak area) or peak height (or peak area) ratios of five or six standard solutions containing a range of solute concentration. The concentration ranges used were from 0 to twice the expected concentration. Each solution was injected at least in duplicate and the data fitted to Eq. 100 using linear regression.

$$y = m.x + c \qquad (100)$$

where y is the peak height (area) or peak height (area) ratio, x is the concentration of the solute, and m and c are regression coefficients.

c. Concentration determination

Two methods were used to determine the concentration of unknown solutions. The first method involved the application of a calibration curve and Eq. 100 as described above. In the second method, the sample responses were compared with the response obtained from one analytical standard of known concentration. The second method was only applied after the linearity of response was confirmed. In both methods the samples and the analytical standards were injected at least in duplicate. In the second method the standard solution was injected after every other sample, and the sample concentrations determined using either Eqs. 101 or 102.

$$c_1 = c_2 \cdot y_1 \cdot y_2^{-1}. \qquad (101)$$

where c refers to the concentration, y refers to the peak height (or area) and the sub-scripts 1 and 2 refer to the sample and the analytical standards respectively.

$$c_1 = c_2 \cdot y_1 \cdot y_2^{-1} \cdot z_1^{-1} \cdot z_2 \qquad (102)$$

where z is the peak height of the internal standard.

2.3.9 Biological fluids and pharmaceutical formulations

a. Biological fluids containing tryptophan

Samples of blood, urine and saliva were taken from healthy male volunteers. The urine was used without pre-treatment, however the saliva and blood were pretreated to remove cellular matter and high molecular weight proteins. The cellular matter of blood was removed by centrifugation at 600 g for 10 minutes and the plasma drawn off with a pasteur pipette. The high molecular weight components of plasma and saliva were removed by centrifugation through a CF 50A Centriflo filter at 2000 g for 20 minutes, as described by Bauman *et al*. (205).

It was found that storage of biological fluids containing tryptophan was not possible (even if frozen at -11°C) due to the rapid degradation of tryptophan. This was consistent with the findings of Grushka *et al.* (206). Consequently all biological samples containing tryptophan were chromatographed within 30 minutes after preparation.

b. 'Tenoretic' tablets

The pharmacologically active components of 'Tenoretic' tablets were extracted either from single tablets or from combined tablet mixes. In both cases the extracting solvent was H_2SO_4:MeCN:H_2O (0.1:25:75). For the single tablet assays, tablets were weighed accurately into 100 ml. standard flasks and about 75 mls. of the extracting solvent added. The tablet was broken up slightly with the aid of a glass rod and then completely dispersed by ultrasonication for 30 minutes. After cooling to room temperature the volume was adjusted to 100 ml with the extracting fluid. Any small quantities of insoluble tablet excipients were removed by filtration through a No. 1 Whatman filter paper.

For the combined tablet mixes, ten tablets were accurately weighed into a glass mortar and ground to a fine powder. Eight aliquots of about 410 mg. of the powder were weighed accurately into 100 ml. standard flasks and extracted as before.

c. 'Inderetic' capsules

The extracting solvent used was H_2SO_4:MeCN:H_2O (0.1:40:60). Ten 'Inderetic' capsules were accurately weighed and the contents transferred to a glass mortar and the empty shells re-weighed. The capsule contents were mixed in the mortar and eight aliquots (155 mg)

accurately weighed into 100 ml. standard flasks. Extracting solvent (75 ml.) was added to the flasks which were then placed in an ultrasonic bath for 30 minutes. The flasks were then cooled to room temperature and the contents adjusted to volume. Any insoluble capsule excipients were filtered off through a No. 1 Whatman filter paper.

2.3.10 Preparation of 12-dien-Zn(II).

12-dien-Zn(II) was prepared from zinc sulphate and 4-dodecyl-diethylenetriamine as described by Karger and co-workers (207). Zinc sulphate (282.5 mg) was weighed into a 50 ml beaker, dissolved in about 25 ml of distilled water. 4-dodecyldiethylenetriamine (271.5mg) was weighed into a second 50 ml. beaker and the $ZnSO_4$ solution added. The combined mixtures were stirred and transferred to a 100 ml. standard flask with distilled water. The flask was shaken gently to avoid excessive foaming and the solution adjusted to volume to produce a 10^{-2} mol.dm^{-3} 12-dien-Zn(II) stock solution. Prolonged storage of the 12-dien-Zn(II) stock solution resulted in slight cloudiness which was prevented by the addition of ammonium acetate (0.13 mol.dm^{-3}).

2.3.11 Synthesis of D,L-carboethoxyheptyltrimethylammonium methyl-sulphonate.

D,L-carboethoxyheptyltrimethylammonium methanesulphonate was synthesised in three stages from D,L-2-aminooctanoic acid, i.e. esterified with ethanol, methylated with formic acid and formaldehyde and finally quaternised with methylmethanesulphonate.

a. Fischer esterification

Absolute alcohol (95 ml.) was saturated with HCl by passage of dry HCl gas. 2-aminooctanoic acid (3.0 gm) was dissolved in the ethanolic HCl and stirred for 18 hours at room temperature in a round bottomed flask. The ethyl-2-aminooctanoate was precipitated by the addition of 6N NaOH (to pH 12) and extracted with diethylether (3 x 100 ml.). The combined ether extracts were dried over $MgSO_4$, filtered and evaporated to dryness at 60°C *in vacuo*, to give a clear yellow oil of ethyl-2-aminooctanoate (1.5 gm, 42.5%). ν_{max} 1720 cm^{-1} (C=O stretch), δ($CDCl_3$, TMS):0.90-1.40 (16H, m, $C\underline{H}_3$, $(C\underline{H}_2)_5C\underline{H}_3$), 1.75(2H, s, $N\underline{H}_2$ disappeared on deuteration), 3.30-3.50(1H, t, C<u>H</u>), 4.00-4.35(2H, q, $C\underline{H}_2$)

b. Eschweiller-Clarke methylation

Ethyl-2-aminooctanoate (1.2 gm.) was refluxed for 5 hours at 85°C with formic acid (3 ml) and aqueous formaldehyde (39%, 4.5 ml.). The tertiary amine [ethyl-2-(N,N-dimethylamino)-octanoate] was precipitated by the addition of 6N NaOH (to pH 12) and extracted with diethylether (3 x 100 ml.). The ether extract was dried over $MgSO_4$, filtered, and evaporated to dryness at 60° *in vacuo* to give a yellow oil of ethyl-2-(N,N-dimethylamino)-octanoate (1.2 gm, 87.0%). ν_{max} 1730 cm^{-1}. δ($CDCl_3$, TMS):0.80-1.40(16H, m, $C\underline{H}_3$, $(C\underline{H}_2)_5\cdot C\underline{H}_3$), 2.30(6H, s, $N(C\underline{H}_3)_2$), 2.90-3.10(1H, t, C<u>H</u>), 3.95-4.35(2H, q, $C\underline{H}_2$).

c. Quaternisation.

Methyl methanesulphonate (100 ml) was purified by distillation at atmospheric pressure from anhydrous K_2CO_3. b.pt.: 199°C (Literature 202°C). (TMS): 3.00(3H, s, $C\underline{H}_3$), 3.90 (3H, s, $C\underline{H}_3$).

Ethyl-2-(N,N-dimethylamino)-octanoate (1.1 gm) was dissolved in sodium dried diethylether (50 ml) and methyl methanesulphonate (3 ml) added dropwise. The reaction mixture was allowed to stand for 30 minutes at room temperature and the diethylether evaporated to dryness at 60°C *in vacuo*. The excess methyl methanesulphonate was removed by heating to 100°C *in vacuo* (5 mm Hg) to give white crystals of D,L-carboethoxyheptyltrimethylammonium methane sulphonate (1.6 gm, 96.2%). ν_{max} 1730 cm^{-1}. δ(TMS, $CDCl_3$) 0.90-1.50(16H, m, $C\underline{H}_3$, $(C\underline{H}_2)C\underline{H}_3$), 2.70(3H, s, $C\underline{H}_3SO_3^-$), 3.40(9H, s, $N(C\underline{H}_3)_3^+$), 4.20-4.40 (2H, q, $C\underline{H}_2$), 2.90-3.10(1H, t, $C\underline{H}$). m.pt.: 58°C.

The analytical sample was obtained by recrystallisation from ethylacetate.

Found: C, 50.2 H, 9.9 N, 4.5

$C_{14}H_{31}NO_5S$ requires C, 51.7, H, 9.5, N, 4.3.

SECTION 3. RESULTS

SECTION 3. RESULTS

3.1 The Effect of Environmental and Constitutional Factors on Solute Capacity Ratios and Functional Group Values.

Table 3.1. The capacity ratios and τ values obtained from a series of benzoic acids under standard conditions. Stationary phase: Spherisorb S5 ODS (Column B), Mobile phase: 50% v/v methanol, 4 x 10^{-4} $mol.dm^{-3}$ C_{13}BDAC, pH 7.5 phosphate buffer. 30 °C.

Solute	κ	τ
BA	1.60	-
2-HBA	2.82	0.25
3-HBA	0.79	-0.30
4-HBA	0.55	-0.45
2-ABA	1.23	-0.11
3-ABA	0.50	-0.50
4-ABA	0.46	-0.55
2-NBA	1.15	-0.14
3-NBA	2.29	0.16
4-NBA	2.24	0.15
2-MBA	1.78	0.05
3-MBA	2.75	0.24
4-MBA	2.57	0.21
2-CBA	1.70	0.03
3-CBA	4.90	0.49
4-CBA	4.47	0.45
CA	3.24	0.31
PAA	1.78	0.05

Table 3.2. The capacity ratios and τ values obtained from a series of azapurinones under standard conditions. Stationary phase: Spherisorb S5 ODS (Column B). Mobile Phase: 50% v/v methanol, 1 x 10^{-4} $mol.dm^{-3}$ C_{11}BDAC, pH 7.5 (2.5 x 10^{-2} K_2HPO_4) 30°C.

Function	κ	τ
H	2.38	-
3-OH	1.66	-0.19
3-NH_2	0.78	-0.48
3-SO_2CH_3	0.48	-0.71
4-SO_2NH_2	0.48	-0.71
3-$SO_2C_3H_7$	1.71	-0.17
3-CN	1.17	-0.31
3-C.$(CH_3)_3$	21.2	0.95
3-O-n-C_4H_9	12.4	0.71
3-$CO_2.C_2H_5$	4.47	0.30
3-SH	2.57	0.04

Table 3.3. The capacity ratios, τ values and logarithms of the reciprocal minimum inhibitory concentrations (MIC) against *S. aureus* (ref. 208) of a series of 1,3,5-s-triazines. Chromatographic conditions: Stationary phase: Spherisorb S5 ODS (Column B). Mobile phase: 50% v/v methanol, 2.6 x 10^{-4} SDDS, pH 2.2 (H_2SO_4 0.1%), 30°C

Group	κ	τ	log (1/MIC) $mol.^{-1}dm^3$
H	2.10	-	2.71
3-OH	0.89	-0.37	
4-OH	0.80	-0.42	
$3\text{-}N(CH_3)_2$	0.66	-0.50	
$4\text{-}NH.CO.CH_3$	1.05	-0.30	
$3\text{-}SO_2CH_3$	0.91	-0.36	
$4\text{-}SO_2NH_2$	0.45	-0.69	
3-CN	1.08	-0.29	2.77
4-CN	0.97	-0.33	2.55
$3\text{-}C.(CH_3)_3$	16.7	0.90	
$4\text{-}C.(CH_3)_3$	18.5	0.95	
$3\text{-}O\text{-}n\text{-}C_6H_{13}$	33.1	1.20	
$3\text{-}n\text{-}C_6H_{13}$	124	1.77	4.34
$4\text{-}n\text{-}C_6H_{13}$	133	1.80	4.94
$3\text{-}SCH_3$	5.05	0.38	
$4\text{-}SCH_3$	4.10	0.29	3.08
$3\text{-}CF_3$	6.25	0.48	3.11
$4\text{-}CF_3$	3.56	0.23	3.11
3-Br	4.18	0.30	2.88
4-Br	3.31	0.20	3.42

Table 3.4. The relationship between the capacity ratios of the substituted benzoic acids and the pairing-ion concentration (C_{13}BDAC) Mobile phase: methanol (50%) in phosphate buffer (KH_2PO_4 4.76×10^{-3} mol.dm^{-3}, Na_2HPO_4 2.02×10^{-3}, pH7.5) + C_{13}BDAC. Stationary phase: Spherisorb S5 ODS (Column B). 30°C.

Benzoic acid	K						
	C_{13}BDAC Concentration mol.dm^{-3} x 10^4						
	0.93	1.86	2.78	3.71	4.64	6.56	7.48
BA	0.33	0.70	1.00	1.43	2.35	2.54	2.06
4-ABA	0.09	0.17	0.18	0.38	0.66	0.80	0.64
3-ABA	0.09	0.17	0.18	0.47	0.73	0.86	0.76
2-ABA	0.31	0.50	0.65	0.65	1.18	2.13	1.64
4-HBA	0.14	0.22	0.35	0.52	0.89	0.91	0.76
3-HBA	0.17	0.33	0.50	0.69	1.18	2.13	1.64
2-HBA	0.73	1.50	2.00	2.54	4.35	4.55	3.30
4-NBA	0.53	1.08	1.35	2.03	3.29	3.43	2.76
3-NBA	0.51	1.19	1.41	2.00	3.31	3.71	2.67
2-NBA	0.17	0.58	0.71	1.05	1.76	1.86	1.55
4-MBA	0.63	1.44	1.59	2.39	3.88	4.05	3.36
3-MBA	0.63	1.42	1.65	2.33	3.95	4.34	3.33
2-MBA	0.40	0.92	1.00	1.63	2.68	2.91	2.27
4-CBA	0.83	2.28	2.82	4.15	6.93	7.54	6.08
3-CBA	0.86	2.34	2.85	4.21	7.26	7.32	5.91
2-CBA	0.35	0.77	0.97	1.06	2.52	2.68	2.20
PAA	0.37	0.89	1.03	1.17	2.52	2.56	1.97
CA	0.56	1.29	2.00	2.05	4.82	5.04	3.78

Table 3.5. τ values obtained from the benzoic acid series at different pairing-ion concentrations (C_{13}BDAC). Conditions as Table 3.4.

Group	τ C_{13}BDAC concentration mol.dm^{-3} x 10^4						
	0.93	1.86	2.78	3.71	4.64	6.56	7.48
4-NH_2	-0.57	-0.62	-0.75	-0.57	-0.55	-0.50	-0.51
3-NH_2	-0.57	-0.62	-0.75	-0.48	-0.51	-0.47	-0.43
2-NH_2	-0.03	-0.13	-0.19	-0.08	-0.10	-0.08	-0.10
4-OH	-0.38	-0.51	0.47	-0.43	-0.42	-0.45	-0.43
3-OH	-0.28	-0.33	-0.30	-0.32	-0.30	-0.28	-0.31
2-OH	0.34	0.33	0.30	0.25	0.27	0.25	0.21
4-NO_2	0.22	0.19	0.13	0.15	0.15	0.13	0.13
3-NO_2	0.21	0.23	0.15	0.15	0.15	0.17	0.13
2-NO_2	-0.28	-0.19	-0.15	-0.14	-0.13	-0.14	-0.12
4-CH_3	0.28	0.31	0.20	0.22	0.22	0.20	0.21
3-CH_3	0.28	0.32	0.22	0.21	0.23	0.23	0.21
2-CH_3	0.08	0.12	0.00	0.05	0.06	0.06	0.04
4-Cl	0.40	0.51	0.45	0.47	0.47	0.47	0.47
3-Cl	0.42	0.52	0.46	0.47	0.49	0.46	0.46
2-Cl	0.04	0.04	-0.01	0.03	0.03	0.02	0.03
CH_2(PAA)	0.05	0.10	0.01	0.07	0.03	0.03	0.02
CH=CH(CA)	0.23	0.36	0.30	0.31	0.31	0.30	0.25

Table 3.6. The capacity ratios and τ values for benzoic acids and sodium cromoglycate (SCG) and their relationship with the number of carbon atoms of the ABDAC pairing-ion alkyl chain. Stationary phase: Spherisorb S5 ODS (Column B), Mobile phase: 50% methanol in phosphate buffer (pH 7.5) + ABDAC (5×10^{-4} $mol.dm^{-3}$).

Solute	Pairing-ion alkyl chain length									
	8		10		12		14		16	
	k	τ	k	τ	k	τ	k	τ	k	τ
3-NBA	0.60	0.18	1.00	0.20	1.82	0.16	3.09	0.17	5.89	0.24
3-MBA	0.71	0.25	1.10	0.24	1.95	0.19	3.39	0.21	6.17	0.26
3-CBA	1.38	0.54	1.95	0.49	3.24	0.41	5.01	0.40	10.7	0.50
BA	0.40	-	0.63	-	1.26	-	2.09	-	3.38	-
SCG	0.51		1.23		4.47		10.7			

Table 3.7. Capacity ratios of 3-nitrobenzoic acid using different concentrations and chain lengths of the ABDAC pairing-ion. Stationary phase: ODS Hypersil (Column G). Mobile phase: 40% methanol in phosphate buffer (pH 7.5) + ABDAC. Temperature 30°C

ABDAC Concentration $mol.dm^{-3} \times 10^3$	ABDAC alkyl chain length			
	10	11	12	14
0.00	0.52	0.60	0.54	0.47
0.10	1.18	1.46	1.99	3.12
0.20	2.72	3.98	5.72	10.6
0.30	2.99	4.95	7.38	16.5
0.40	3.30	6.03	10.2	19.6
0.50	3.82	6.78	10.5	22.5
0.75	4.46	8.08	11.2	24.5
1.00	5.52	8.54	13.3	27.9

Table 3.8. The logarithm of the capacity ratios, κ, and τ values of the benzoic acids with change in propan-2-ol volume fraction, ϕ. Stationary phase: ODS Hypersil (Column G). Mobile phase: Propan-2-ol-water, 5×10^{-4} mol.dm^{-3} C_{14}BDAC, pH 7.5 (2.5×10^{-2} K_2HPO_4). 30°C

Function	Propan-2-ol composition (ϕ)									
	0.200		0.225		0.250		0.255		0.300	
	log κ	τ	log κ	τ	log κ	τ	log κ	τ	log κ	τ
BA	1.19	-	0.93	-	0.70	-	0.58	-	0.10	-
4-ABA	0.16	-1.03	0.05	-0.88	-0.09	-0.79	-0.18	-0.76	-0.45	-0.55
3-ABA	0.45	-0.74	0.28	-0.65	0.09	-0.61	0.02	-0.56	-0.36	-0.46
2-ABA	1.12	-0.07	0.86	-0.07	0.59	-0.11	0.52	-0.06	0.00	-0.10
4-HBA	0.61	-0.58	0.40	-0.53	0.16	-0.54	0.10	-0.48	-0.30	-0.40
3-HBA	0.95	-0.24	0.68	-0.25	0.43	-0.27	0.34	-0.24	-0.17	-0.27
4-NBA	1.56	0.37	1.25	0.32	0.96	0.26	0.84	0.26	0.27	0.17
3-NBA	1.54	0.35	1.23	0.30	0.95	0.25	0.84	0.26	0.24	0.14
2-NBA	0.96	-0.23	0.73	-0.20	0.50	-0.20	0.38	-0.20	-0.09	-0.19
4-MBA	1.58	0.39	1.30	0.37	1.05	0.35	0.89	0.31	0.36	0.26
3-MBA	1.56	0.37	1.29	0.36	1.04	0.34	0.89	0.31	0.34	0.24
2-MBA	1.13	-0.06	0.92	-0.01	0.71	0.01	0.60	0.02	0.12	0.02
4-CBA					1.25	0.55	1.12	0.54	0.62	0.52
3-CBA					1.26	0.56	1.13	0.55	0.62	0.52
2-CBA	1.17	-0.02	0.93	0.00	0.71	0.01	0.64	0.06	0.12	0.02

Table 3.9. The logarithm of the capacity ratios, κ, and τ values of the benzoic acids with change in methanol volume fraction, ϕ, Stationary phase: ODS Hypersil (Column G), Mobile Phase: methanol-water, 5×10^{-4} mol.dm^{-3} C_{14}BDAC, pH 7.5 (2.5×10^{-2} mol.dm^{-3} K_2HPO_4), 30°C

Function	Methanol composition ϕ							
	0.34		0.40		0.50		0.60	
	log κ	τ	log κ	τ	log κ	τ	log κ	τ
BA	1.37	-	0.98	-	0.58	-	0.10	-
4-ABA	0.35	-1.02	0.14	-0.84	-0.17	-0.75	-0.49	-0.59
3-ABA	0.66	-0.69	0.37	-0.61	0.00	-0.58	-0.35	-0.45
2-ABA	1.35	-0.02	0.97	-0.01	0.49	-0.09	-0.03	-0.13
4-HBA	0.83	-0.54	0.45	-0.53	0.04	-0.54	-0.29	-0.39
3-HBA	1.18	-0.15	0.74	-0.24	0.28	-0.30	-0.16	-0.26
2-HBA	1.90	0.53	1.56	0.58	0.93	0.65	0.30	0.40
4-NBA	1.65	0.28	1.23	0.25	0.75	0.17	0.22	0.12
3-NBA	1.70	0.33	1.27	0.29	0.80	0.22	0.23	0.13
2-NBA	1.25	-0.12	0.88	-0.10	0.49	-0.09	0.04	-0.06
4-MBA	1.76	0.39	1.33	0.35	0.88	0.30	0.33	0.23
3-MBA	1.81	0.44	1.36	0.38	0.91	0.33	0.34	0.24
2-MBA	1.48	0.11	1.04	0.06	0.66	0.08	0.20	0.10
4-CBA	2.10	0.73	1.63	0.65	1.11	0.53	0.47	0.37
3-CBA	2.10	0.73	1.64	0.66	1.13	0.55	0.50	0.40
2-CBA	1.42	0.05	1.04	0.06	0.65	0.07	0.15	0.05

Table 3.10. The logarithm of the capacity ratios, κ, and τ values of the benzoic acids with change in acetonitrile volume fraction, ϕ. Stationary phase: ODS Hypersil (Column G). Mobile phase: acetonitrile-water, 5×10^{-4} C_{14}BDAC, pH 7.5 (2.5×10^{-2} mol.dm^{-3} K_2HPO_4). 30°C

Function	Acetonitrile composition ϕ									
	0.200		0.250		0.275		0.300		0.350	
	log κ	τ	log κ	τ	log κ	τ	log κ	τ	log κ	τ
BA	1.31	-	0.85	-	0.58	-	0.38	-	0.04	-
4-ABA	0.29	-1.02	0.11	-0.74	0.00	-0.58	-0.14	-0.52	-0.34	-0.38
3-ABA	0.59	-0.72	0.30	-0.55	0.13	-0.45	-0.03	-0.41	-0.26	-0.30
2-ABA	1.32	0.01	0.89	0.04	0.59	0.01	0.39	0.01	0.05	0.01
4-HBA	0.59	-0.72	0.29	-0.56	0.10	-0.48	-0.03	-0.41	-0.25	-0.29
3-HBA	1.04	-0.27	0.58	-0.27	0.34	-0.24	0.15	-0.23	-0.15	-0.19
4-NBA			1.24	0.39	0.97	0.39	0.72	0.34	0.37	0.33
3-NBA			1.27	0.42	0.94	0.36	0.71	0.33	0.34	0.30
2-NBA			0.86	0.01	0.64	0.06	0.42	0.04	0.13	0.09
4-MBA			1.18	0.33	0.89	0.31	0.65	0.27	0.23	0.19
3-MBA			1.22	0.37	0.92	0.34	0.63	0.25	0.63	0.22
2-MBA			0.85	0.00	0.60	0.02	0.40	0.02	0.05	0.01
4-CBA			1.50	0.65	1.15	0.57	0.91	0.53	0.42	0.38
3-CBA			1.52	0.67	1.18	0.60	0.91	0.53	0.46	0.42
2-CBA			0.90	0.05	0.67	0.09	0.45	0.07	0.03	-0.01

Table 3.11. The logarithm of the capacity ratios, κ, and τ values of the benzoic acids with change in tetrahydrofuran volume fraction, ϕ. Stationary phase: ODS Hypersil (Column G). Mobile phase: Tetrahydrofuran-water, 5×10^{-4} mol. dm^{-3} C_{14}BDAC, pH 7.5 (K_2HPO_4 2.5×10^{-2} mol.dm^{-3}). 30°C.

Function	Tetrahydrofuran composition (ϕ)									
	0.150		0.200		0.250		0.265		0.300	
	log κ	τ	log κ	τ	log κ	τ	log κ	τ	log κ	τ
BA	1.46	-	1.19	-	0.70	-	0.58	-	0.03	-
4-ABA	0.36	-1.10	0.23	-0.96	-0.03	-0.73	-0.17	-0.75	-0.68	-0.71
3-ABA	0.72	-0.74	0.52	-0.67	0.15	-0.55	0.03	-0.55	-0.42	-0.45
2-ABA	1.51	0.05	1.25	0.06	0.73	0.03	0.50	0.08	0.06	0.03
4-HBA	0.93	-0.47	0.73	-0.36	0.34	-0.36	0.20	-0.38	-0.19	-0.22
3-HBA	1.38	-0.08	1.09	-0.10	0.58	-0.22	0.36	-0.22	-0.07	-0.10
4-NBA					1.33	0.53	1.20	0.62	0.59	0.56
3-NBA					1.33	0.53	1.16	0.58	0.48	0.45
2-NBA	1.33	-0.13	1.08	-0.11	0.63	-0.07	0.50	-0.06	-0.02	-0.05
4-MBA					0.98	0.28	0.86	0.28	0.26	0.23
3-MBA					0.93	0.23	0.83	0.25	0.23	0.20
2-MBA	1.34	-0.12	1.10	-0.09	0.65	-0.05	0.50	-0.08	-0.03	-0.06
4-CBA					1.26	0.56	1.15	0.57	0.63	0.60
3-CBA					1.25	0.55	1.15	0.57	0.62	0.59
2-CBA	1.40	-0.06	1.15	-0.04	0.65	-0.05	0.52	-0.06	-0.03	-0.06

Table 3.12. The logarithm of the capacity ratios, κ , of the 1,3,5-s-triazines with change in acetonitrile mobile phase composition. Stationary phase: ODS Hypersil (Column F). Mobile phase: acetonitrile:water, 0.1% H_2SO_4, 5 x 10^{-4} $mol.dm^{-3}$ SDDS, (pH 2.2). Temperature 30°C

Triazine Substituent	Log κ										
	Acetonitrile % v/v										
	20	24	28	32	36	40	50	60	70	80	96
3-OH	1.44	0.96	0.65	0.35	0.08						
4-OH	1.42	0.88	0.58	0.30	0.07						
4-$NHCOCH_3$	1.49	0.97	0.68	0.36	0.04						
4-$N(CH_3)_2$		1.60	1.24	0.71	0.40	0.24	0.26	0.27	0.28	0.24	
3-SO_2CH_3	1.25	0.83	0.58	0.21	0.08						
4-SO_2NH_2	1.03	0.63	0.39	0.13	-0.06						
3-CN	1.50	1.05	0.79	0.49	0.20						
3-$C(CH_3)_3$			1.98	1.51	1.01	0.82	0.59	0.56	0.41	0.23	
3-O-n-C_5H_{13}						1.38	1.02	1.06	0.82	0.58	
3-O-n-C_9H_{19}						1.80	1.48	1.30	1.02	0.84	
3-SCH_3		1.76	1.40	0.99	0.60	0.43	0.32	0.29	0.18	0.04	
3-CF_3		1.81	1.46	1.07	0.66	0.44	0.33	0.25	0.14	-0.03	
3-Br		1.71	1.35	0.99	0.59						
3-$(CH_2)_4$-phenyl						1.37	1.02	0.88	0.60	0.31	
3-$(CH_2)_4$-phenyl-4'-OCH_3						1.42	1.09	1.00	0.71	0.39	
H	1.81	1.27	0.93	0.61	0.31	0.18	0.14	0.15	0.10	-0.05	-0.18

Table 3.13. τ values obtained from the 1,3,5-s-triazines at different acetonitrile concentrations. Conditions as Table 3.12.

Group	τ									
	Acetonitrile concentration % v/v									
	20	24	28	32	36	40	50	60	70	80
3-OH	-0.37	-0.31	-0.28	-0.26	-0.23					
4-OH	-0.39	-0.39	-0.35	-0.21	-0.24					
4-NHCOCH	-0.32	-0.30	-0.25	-0.25	-0.27					
$3\text{-}SO_2CH_3$	-0.56	-0.44	-0.35	-0.40	-0.23					
$4\text{-}SO_2NH_2$	-0.78	-0.64	-0.54	-0.48	-0.37					
3-CN	-0.31	-0.22	-0.14	-0,12	-0.11					
$3\text{-}C(CH_3)_3$			1.05	0.90	0.80	0.64	0.45	0.41	0.31	0.28
$3\text{-}O\text{-}n\text{-}C_6H_{13}$						1.20	0.88	0.89	0.72	0.63
$3\text{-}O\text{-}n\text{-}C_9H_{19}$						1.62	1.34	1.15	0.92	0.89
$3\text{-}SCH_3$		0.49	0.47	0.38	0.29	0.25	0.18	0.14	0.08	0.09
$3\text{-}CF_3$		0.54	0.53	0.46	0.35	0.26	0.19	0.10	0.04	0.02
3-Br		0.44	0.42	0.38	0.28					
$3\text{-}(CH_2)_4$-phenyl						1.15	0.88	0.73	0.50	0.36
$3\text{-}(CH_2)_4$-phenyl-4'-OCH_3						1.24	0.95	0.85	0.61	0.44

Table 3.14. The logarithm of the capacity ratios, κ, of the 1,3,5-s-triazines with change in methanol composition. Stationary phase: ODS Hypersil (Column F). Mobile phase: SDDS (5×10^{-4} mol.dm^{-3}) in methanol:water 0.1% H_2SO_4 (pH 2.2). Temperature 30°C

Triazine Substituent	log κ Methanol % v/v						
	30	40	44	60	70	80	96
4-OH		0.87	0.63	-0.04	-0.26		
4-$NHCOCH_3$	1.70	1.04	0.75	0.08	-0.14	-0.26	-0.32
3-SO_2CH_3	1.38	0.77	0.55	-0.04			
4-SO_2NH_2	0.94	0.48	0.25	-0.25	-0.50		
3-$C(CH_3)_3$				0.87	0.41	-0.07	-0.32
3-O-n-C_6H_{13}				1.33	0.88	0.44	-0.12
3-O-n-C_9H_{19}						0.76	-0.04
3-SCH_3				0.44	0.07	-0.10	-0.32
3-Br		1.60	1.34	0.46	0.12	-0.07	-0.26
H	2.00	1.25	1.00	0.33	-0.02	-0.16	-0.30

Table 3.15. τ values obtained from the 1,3,5-s-triazines at different methanol concentrations. Conditions as Table 3.14.

Group	τ Methanol concentration % v/v						
	30	40	44	60	70	80	96
4-OH		-0.38	-0.37	-0.37	-0.24		
4-$NHCOCH_3$	-0.30	-0.29	-0.25	-0.21	-0.12	-0.10	-0.02
3-SO_2CH_3	-0.62	-0.48	-0.45	-0.37			
4-SO_2NH_2	-1.06	-0.77	-0.75	-0.58	-0.52		
3-$C(CH_3)_3$				0.54	0.43	0.09	0.02
3-O-n-C_6H_{13}				1.00	0.90	0.60	0.18
3-O-n-C_9H_{19}						0.92	0.26
3-SCH_3				0.11	0.09	0.06	0.02
3-Br		0.35	0.34	0.13	0.14	0.09	0.04

Table 3.16. The capacity ratios, κ, of the benzoic acids at different mobile phase ionic strengths (adjusted with KNO_3). Stationary phase: Spherisorb S5 ODS (Column B). Mobile phase: 20% v/v acetonitrile, 3.5 x10^{-4} mol.dm^{-3} C_{13}BDAC, pH 7.5 30°C

Solute	κ					
	Ionic strength mol.dm^{-3} x 10^2					
	0.05	0.25	0.46	0.86	1.67	4.08
BA	60.2	48.8	34.5	23.9	15.4	7.67
4-ABA	13.0	11.5	7.65	5.75	3.42	1.50
3-ABA	17.3	13.4	9.90	7.40	4.39	2.00
2-ABA	53.0	45.6	32.5	26.4	15.8	7.89
4-HBA	16.7	15.5	10.2	7.00	4.56	2.22
3-HBA	24.0	22.8	15.1	11.4	7.03	3.52
2-NBA	39.0	36.7	25.9	17.3	10.6	6.56
4-NBA			72.3	55.0	32.4	18.2
3-NBA			75.9	57.4	32.9	18.8

Table 3.17. τ values at different mobile phase ionic strength (adjusted with KNO_3). Conditions as Table 3.16.

Group	τ					
	Ionic strength $mol.dm^{-3} \times 10^2$					
	0.05	0.25	0.46	0.86	1.67	4.08
4-NH_2	-0.67	-0.63	-0.65	-0.62	-0.65	-0.71
3-NH_2	-0.54	-0.56	-0.54	-0.53	-0.55	-0.58
2-NH_2	-0.06	-0.03	-0.03	0.04	0.01	0.07
4-OH	-0.56	-0.50	-0.53	-0.53	-0.53	-0.54
3-OH	-0.40	-0.33	-0.36	-0.32	-0.34	-0.39
2-NO_2	-0.18	-0.12	-0.13	-0.14	-0.16	-0.14
3-NO_2			0.32	0.38	0.32	0.37
4-NO_2			0.34	0.36	0.33	0.39

Table 3.18. The capacity ratios, κ, of the benzoic acids at different mobile phase ionic strengths (adjusted with K_2HPO_4). Stationary phase: Spherisorb S5 ODS (Column B). Mobile phase: 20% acetonitrile, 3.5×10^{-4} C_{13}BDAC, pH 7.5. 30°C.

Solute	κ				
	Ionic strength $mol.dm^{-3} \times 10^2$				
	0.05	0.51	1.21	2.37	4.69
BA	60.2	16.5	8.90	6.53	5.64
4-ABA	13.0	4.00	1.73	1.20	1.07
3-ABA	17.3	5.58	2.64	1.80	1.22
2-ABA	53.0	17.3	9.91	7.68	5.87
4-HBA	16.7	5.83	2.62	2.00	1.41
3-HBA	24.0	7.85	2.62	3.30	2.66
4-NBA			23.3	16.5	14.2
3-NBA			22.9	18.0	14.4
2-NBA	39.0	13.2	7.19	5.00	4.00

Table 3.19. τ values at different ionic strengths (adjusted with K_2HPO_4). Conditions as Table 3.18.

Group	τ				
	Ionic strength mol.dm^{-3} x10^2				
	0.05	0.51	1.21	2.37	4.69
4-NH_2	-0.67	-0.62	-0.71	-0.74	-0.72
3-NH_2	-0.54	-0.47	-0.53	-0.56	-0.66
2-NH_2	-0.06	0.02	0.05	-0.07	-0.02
4-OH	-0.56	-0.45	-0.53	-0.51	-0.60
3-OH	-0.40	-0.32	-0.30	-0.30	-0.33
4-NO_2			0.41	0.44	0.40
3-NO_2			0.41	0.44	0.41
2-NO_2	-0.18	-0.10	-0.09	-0.12	-0.15

Table 3.20. The capacity ratios, κ, of the benzoic acids at different temperatures. Stationary phase: Spherisorb S5 ODS (Column B). Mobile phase: 20% acetonitrile, 5×10^{-4} mol.dm^{-3} C_{13}BDAC, pH 7.5 (0.025 mol.dm^{-3} K_2HPO_4).

Solute	κ				
	Temperature ^{o}C				
	18.8	31.0	38.2	50.6	60.5
BA	28.8	14.8	11.7	7.53	5.00
4-ABA	3.17	2.22	1.95	1.43	1.20
2-ABA	28.5	14.1	10.6	6.65	4.17
4-HBA	5.80	3.33	2.80	2.03	1.45
3-HBA	10.8	6.10	4.95	3.43	2.40
4-NBA		31.9	25.1	15.3	9.33
2-NBA		9.20	8.00	5.67	4.07
4-MBA		33.1	24.5	15.0	9.12
2-MBA		12.7	11.0	7.60	5.89
4-CBA		75.1	53.0	31.0	13.4
2-CBA		11.7	10.2	7.20	5.00
CA		37.7	29.5	18.1	12.8

Table 3.21. τ values at different temperatures. Conditions as Table 3.20

Group	τ				
	Temperature °C				
	18.8	31.0	38.2	50.6	60.5
4-NH_2	-0.96	-0.82	-0.78	-0.72	-0.62
2-NH_2	-0.01	-0.02	-0.04	-0.06	-0.08
4-OH	-0.70	-0.65	-0.62	-0.57	-0.54
3-OH	-0.43	-0.39	-0.37	-0.34	-0.32
4-NO_2		0.33	0.32	0.30	0.27
2-NO_2		-0.21	-0.17	-0.13	-0.09
4-CH_3		0.35	0.32	0.30	0.26
2-CH_3		-0.08	-0.03	0.00	0.07
4-Cl		0.71	0.65	0.61	0.58
2-Cl		-0.10	-0.06	-0.02	0.00
CH=CH		0.41	0.40	0.38	0.36

Table 3.22 The capacity ratios, κ, of the benzoic acids at different temperatures. Stationary phase: SAS Hypersil (Column I). Mobile phase: 25% v/v acetonitrile, 10^{-3} mol.dm^{-3} C_{14}BDAC, 0.13 mol.dm^{-3} NH_4 Acetate, pH 7.1 30°C.

Solute	κ			
	Temperature °C			
	25.0	36.3	45.4	54.4
BA	6.91	5.69	4.52	3.72
4-ABA	1.23	1.07	0.93	0.87
4-MBA	14.8	11.3	8.78	7.10
2-PA	8.00	6.05	5.31	4.28
3-PA	8.81	6.52	5.12	3.99
4-PA	5.30	4.17	3.52	2.97

Table 3.23. The capacity ratios, κ, of the benzoic acids at different temperatures. Stationary phase: SAS Hypersil (Column I). Mobile phase: 25% v/v acetonitrile, 10^{-3} mol.dm^{-3} 12-dien-Zn(II) 0.13 mol.dm^{-3} NH_4 Acetate, pH 7.1. 30°C

Solute	κ			
	Temperature °C			
	23.7	33.5	45.7	55.2
BA	12.9	10.5	7.83	8.46
4-ABA	3.22	2.73	2.15	1.86
4-MBA	33.6	25.5	18.8	14.7
2-PA	113	84.0	52.8	37.2
3-PA	30.9	22.2	14.4	10.5
4-PA	12.9	9.76	6.73	5.27

Table 3.24. τ values at different temperatures. conditions as Table 3.22.

Group	τ Temperature °C			
	25.0	36.3	45.4	54.4
4-NH_2	-0.75	-0.72	-0.68	-0.63
4-CH_3	0.33	0.30	0.29	0.28
2-COOH	0.06	0.07	0.08	0.08
3-COOH	0.10	0.06	0.06	0.03
4-COOH	-0.12	-0.13	-0.10	-0.10

Table 3.25. τ values at different temperatures, conditions as Table 3.23

Group	τ Temperature °C			
	23.7	33.5	45.7	55.2
4-NH_2	-0.60	-0.58	-0.56	-0.54
4-CH_3	0.42	0.39	0.38	0.36
2-COOH	1.06	0.90	0.83	0.76
3-COOH	0.38	0.33	0.27	0.21
4-COOH	0.00	-0.03	-0.06	-0.09

Table 3.26. The capacity ratios, κ, of the benzoic acids using five different stationary phases. Mobile phase: 50% v/v methanol in phosphate buffer, 5 x 10^{-4} mol.dm^{-3} C_{14}BDAC, pH 7.5, temperature: 30°C.

Benzoic Acid	κ stationary phase				
	Partisil 10 ODS	Spherisorb hexyl	Spherisorb S5 ODS	ODS Hypersil	Partisil 10 ODS2
BA	1.3	2.6	2.8	3.8	10.2
PAA	1.5	2.8	2.8	4.0	10.0
CA	2.9	2.2	5.1	8.5	21.4
2-HBA	2.1	5.2	5.5	8.5	24.0
3-HBA	0.65	1.4	1.3	1.9	3.1
4-HBA	0.89	0.89	1.0	1.1	2.1
2-ABA	1.2	2.1	2.8	3.1	8.3
3-ABA	0.46	0.89	0.87	1.0	1.9
4-ABA	0.35	0.66	0.54	0.74	1.3
2-NBA	1.1	2.2	1.7	3.1	6.0
3-NBA	2.2	3.5	4.1	6.3	15.8
4-NBA	2.0	3.3	3.9	5.6	15.8
2-CBA	1.2	3.1	2.3	4.5	8.5
3-CBA	4.7	7.4	8.1	13.5	36.3
4-CBA	4.7	7.2	7.9	12.9	38.0
2-MBA	1.5	3.4	2.7	4.9	11.0
3-MBA	2.6	5.2	4.7	8.1	21.9
4-MBA	2.6	5.0	4.6	7.6	20.4

Table 3.27. τ values using five different stationary phases. Conditions as Table 3.26

Function	τ				
	Stationary phase				
	Partisil 10 ODS	Spherisorb hexyl	Spherisorb S5 ODS	ODS Hypersil	Partisil 10 ODS2
2-OH	0.21	0.30	0.30	0.35	0.37
3-OH	-0.30	-0.28	-0.34	-0.30	-0.52
4-OH	-0.17	-0.37	-0.44	-0.54	-0.67
$2\text{-}NH_2$	-0.03	-0.10	0.00	-0.09	-0.09
$3\text{-}NH_2$	-0.45	-0.37	-0.49	-0.58	-0.72
$4\text{-}NH_2$	-0.58	-0.60	-0.70	-0.71	-0.89
$2\text{-}NO_2$	-0.05	-0.08	-0.19	-0.09	-0.22
$3\text{-}NO_2$	0.22	0.12	0.17	0.22	0.19
$4\text{-}NO_2$	0.18	0.11	0.16	0.17	0.19
2-Cl	-0.03	-0.02	-0.07	-0.07	-0.07
3-Cl	0.55	0.45	0.55	0.55	0.55
4-Cl	0.55	0.44	0.47	0.53	0.57
$2\text{-}CH_3$	0.06	0.11	0.00	0.11	0.03
$3\text{-}CH_3$	0.30	0.30	0.24	0.33	0.33
$4\text{-}CH_3$	0.31	0.28	0.22	0.30	0.31
CH_2	0.07	0.02	0.00	0.02	0.01
CH=CH	0.34	0.33	0.27	0.35	0.32

Table 3.28. The capacity ratios, κ, of the benzoic acids at different mobile phase pH. Stationary phase: Spherisorb S5 ODS (Column B). Mobile phase: 10% acetonitrile, 0.04 mol.dm^{-3} KNO_3/HNO_3, 5 x 10^{-4} mol.dm^{-3} C_{13}BDAC. Temperature 29°C

Benzoic acid	pH				
	2.00	3.05	4.20	6.00	7.00
BA	5.10	9.80	14.5	21.8	23.0
4-ABA	1.10	1.65	2.30	3.25	3.40
3-ABA	0.40	1.05	2.60	4.35	6.50
2-ABA	3.80	5.60	8.60	17.6	22.5
4-HBA	1.50	2.83	4.15	6.15	6.63
3-HBA	2.20	4.05	6.90	10.2	12.6
4-MBA	12.8	18.9	34.0	54.7	56.2

Table 3.29. τ values at different mobile phase pH. Conditions as Table 3.28

Function	τ				
	pH				
	2.00	3.05	4.20	6.00	7.00
4-NH_2(NH_3^+)	-0.67	-0.77	-0.80	-0.83	-0.83
3-NH_2(NH_3^+)	-1.11	-0.97	-0.75	-0.70	-0.58
2-NH_2(NH_3^+)	-0.13	-0.24	-0.23	-0.09	-0.01
3-OH	-0.37	-0.38	-0.32	-0.33	-0.36
4-OH	-0.53	-0.54	-0.54	-0.55	-0.54
4-CH_3	0.40	0.29	0.37	0.40	0.39

Table 3.30. The capacity ratios, κ, of the amino acids at different mobile phase pH. Stationary phase: Spherisorb S5 ODS (Column J). Mobile phase: 10% methanol, K_2HPO_4/H_3PO_4 (0.025 mol.dm^{-3}), temperature 30°C.

Solute	κ								
	pH								
	2.00	3.00	3.50	4.00	4.50	5.00	5.50	6.00	6.50
His	0.15	0.08	0.08	0.13	0.12	0.15	0.21	0.25	0.21
DOPA	1.19	1.72	0.54	0.45	0.50	0.50	0.54	0.59	0.59
Phe	3.44	2.99	1.90	1.66	1.63	1.58	1.73	1.85	1.70
CPA	10.6	11.5	6.58	5.59	5.38	5.53	5.66	5.91	5.70
5-HTP	4.35	3.40	2.32	1.81	1.81	1.88	1.94	2.04	1.88
Trp	15.0	8.10	4.60	3.81	3.78	3.85	3.88	3.91	3.72
4-ABA	1.89	2.89	4.70	4.34	2.75	2.00	1.24	0.56	0.25
3-ABA	1.24	1.28	1.89	1.83	1.56	0.97	0.65	0.47	0.16
2-ABA	4.58	7.35	9.15	8.81	6.52	4.39	2.93	1.50	0.63

Table 3.31. The capacity ratios, κ, for amino acids at different mobile phase pH. Stationary phase: Spherisorb S5 ODS (Column J). Mobile phase: 10% methanol, K_2HPO_4/H_3PO_4 (2.5×10^{-2} mol.dm^{-3}. 5×10^{-4} mol.dm^{-3} SDDS. Temperature 30°C.

Solute	κ					
	pH					
	2.00	3.00	4.00	5.00	6.00	7.00
His	6.63	5.75	2.96	2.60	2.08	0.61
DOPA	5.44	3.27	0.86	0.85	0.85	1.06
Phe	*	14.2	2.89	2.23	2.12	1.06
CPA	*	*	12.4	8.04	7.62	5.59
5-HTP	*	16.1	3.34	2.18	1.97	1.70
Trp	*	*	13.1	5.53	4.77	3.47
4-ABA	3.89	3.71	3.08	0.76	0.61	0.30
3-ABA	6.20	4.94	1.11	0.54	0.40	0.21
2-ABA	9.63	10.1	7.03	3.14	0.69	0.48

* not eluted under these conditions.

Table 3.32. The capacity ratios, κ, for amino acids at different mobile phase pH. Stationary phase: Spherisorb S5 ODS (Column J). Mobile phase: 10% methanol, K_2HPO_4 (2.5×10^{-2}), H_3PO_4, 5×10^{-4} mol.dm^{-3} C_{11}BDAC. Temperature 30°C.

	pH					
Solute	2.00	3.00	4.00	5.00	6.00	7.00
His	0.16	0.06	0.08	0.07	0.13	0.12
DOPA	0.91	0.65	0.68	0.56	0.70	0.79
Phe	2.05	1.52	1.19	1.15	1.89	2.01
CPA	7.00	4.70	4.88	4.94	5.34	5.67
5-HTP	2.51	1.75	1.89	1.98	2.08	2.21
Trp	6.24	4.70	3.79	3.94	4.12	4.36
4-ABA	1.78	2.94	5.41	6.89	6.90	3.88
3-ABA	0.95	1.33	6.41	14.5	15.3	13.1
2-ABA	4.99	8.23	13.1	*	*	*

* not eluted under these conditions.

Table 3.33. The capacity ratios, κ, of the acidic metabolites of histidine phenylalanine and tryptophan at different pairing-ion concentrations (C_{14}BDAC). Stationary phase: ODS Hypersil (Column G), mobile phase: 20% acetonitrile, 2.5 x 10^{-2} mol. dm^{-3} K_2HPO_4, pH 7.5. Temperature 30°C.

Solute	κ					
	C_{14}BDAC mol.dm^{-3}x10^4					
	0.00	1.00	2.00	3.00	4.00	5.00
IAA	0.05	0.07	0.19	0.30	0.34	0.36
UA	0.06	0.11	0.27	0.58	0.83	0.84
HPPA	0.26	0.69	2.04	2.82	9.20	9.96
HMMA	0.09	0.23	0.56	1.40	2.37	2.44
HMA	0.08	0.28	0.76	2.15	3.54	4.06
HG	0.22	0.60	2.09	5.17	10.2	11.2
5-HIAA	0.21	0.54	1.05	2.81	5.03	5.55
HAA	0.10	0.39	0.80	2.34	4.20	4.60

Table 3.34. The capacity ratios, κ, of the acidic metabolites of histidine phenylalanine and tryptophan at different pairing-ion concentrations (C_{14}BDAC). Stationary phase: ODS Hypersil (Column G). Mobile phase: 15% acetonitrile, 2.5 x 10^{-2} K_2HPO_4 + C_{14}BDAC, pH 7.5. Temperature 30°C.

Solute	κ					
	C_{14}BDAC mol.dm^{-3} x 10^4					
	0.00	1.00	2.00	3.00	4.00	5.00
UA	0.06	0.15	0.33	0.77	0.98	1.18
IAA	0.04	0.07	0.17	0.39	0.46	0.47
HPPA	0.36	1.63	6.71	12.5	21.1	30.2
HMMA	0.09	0.40	1.27	3.61	3.80	4.55
HMA	0.07	0.48	2.32	4.33	9.05	14.2
HG	0.29	1.42	6.13	11.6	25.7	33.3
5-HIAA	0.33	2.33	3.26	6.24	8.95	10.9
HAA	0.14	0.64	2.22	4.13	7.34	10.2

Table 3.35. The capacity ratios, κ, of the acidic metabolites of histidine, phenylalanine and tryptophan at different pairing-ion concentrations (C_{14}BDAC). Stationary phase: ODS Hypersil (Column G). Mobile phase: 25% acetonitrile, 2.5 x 10^{-2} mol.dm^{-3} K_2HPO_4, + C_{14}BDAC, pH 7.5. Temperature 30°C.

Solute	κ					
	C_{14}BDAC mol.dm^{-3} x 10^4					
	0.00	1.00	2.00	3.00	4.00	5.00
IAA	0.03	0.13	0.19	0.24	0.26	0.29
UA	0.05	0.13	0.32	0.49	0.61	0.64
HPPA	0.31	0.53	1.65	2.10	4.00	4.50
HMMA	0.07	0.28	0.74	1.09	1.29	1.36
HMA	0.07	0.28	0.76	1.17	1.36	1.42
HG	0.11	0.32	0.84	1.24	1.51	1.56
5-HIAA	0.10	0.31	1.30	1.85	2.18	2.38
HAA	0.11	0.30	1.07	1.47	2.19	2.38

Table 3.36.' The capacity ratios, κ of the acidic metabolites of histidine, phenylalanine and tryptophan at different acetonitrile concentrations. Stationary phase: ODS Hypersil (Column G). Mobile phase: 10^{-3} mol.dm^{-3} 12-dien-Zn(II), 0.13 mol.dm^{-3} NH_4Ac, pH 7.1. Temperature: 30°C.

Solute	κ Acetonitrile %v/v		
	30	25	20
IAA	5.63	7.85	13.4
UA	2.51	4.01	9.00
HPPA	1.57	2.45	4.85
5-HIAA	4.05	5.80	13.0
HAA	3.50	12.2	23.3
HMA	1.42	2.19	5.55
HMMA	1.38	1.98	3.55
HG	2.54	4.42	8.79

Table 3.37. The capacity ratios of tryptophan at different mobile phase concentrations of SDS, SDDS and STDS pairing-ions. Stationary phase: Spherisorb S5 ODS (Column A). Mobile phase: Methanol:water (1:1), pH 2.25 (citrate buffer). 30°C.

SDS		SDDS		STDS	
Concentration $(mol.dm^{-3})x10^{4}$	K	Concentration $(mol.dm^{-3}x10^{4}$	K	Concentration $(mol.dm^{-3}x10^{4}$	K
0.49	0.95	0.99	1.95	0.29	1.06
1.23	1.25	1.99	2.75	0.64	1.87
2.45	1.36	2.98	3.19	1.29	2.86
3.68	2.00	3.98	3.52	1.60	3.17
4.91	2.30	4.97	3.61	4.82	5.69

Table 3.38. The capacity ratios, κ, of atenolol, I, and five structurally related analogues, II to VI, at different pairing-ion concentrations (SOS). Stationary phase: ODS Hypersil (Column G). Mobile phase: 0.1% v/v H_2SO_4
(a). 30% v/v methanol, (b). 16% v/v acetonitrile. 30°C.
Key as Fig. 2.2.

(a)

Analogue	κ					
	SOS concentration $mol.dm^{-3} \times 10^3$					
	0.00	0.40	0.60	0.75	1.00	1.50
I	0.46	1.58	1.95	2.23	2.87	3.22
II	1.23	4.18	5.07	5.73	7.21	8.62
III	1.35	4.60	5.71	6.44	7.99	9.73
IV	1.90	1.74	1.73	1.75	1.79	1.73
V	5.90	5.06	4.94	4.90	4.82	4.73
VI	3.48	3.19	3.09	3.09	3.26	2.95

(b)

Analogue	κ					
	SOS concentration $mol.dm^{-3} \times 10^3$					
	0.00	0.25	0.50	0.75	1.00	1.50
I	0.47	1.29	2.03	2.85	2.82	3.74
II	2.16	5.47	8.57	11.3	12.3	16.0
III	2.55	7.39	11.3	15.5	16.0	21.1
IV	2.50	2.36	2.45	2.41	2.30	2.38
V	5.15	4.47	4.72	4.49	4.65	4.72
VI	3.63	3.37	3.57	3.57	3.46	3.42

Table 3.39. The capacity ratios, κ, of sodium 4-hydroxymandelate, SHM, and 6-chlorocinnoline-3-carboxylic acid, CCA, at different pairing-ion concentrations (C_{14}BDAC). Stationary phase: ODS Hypersil (Column G). Mobile phase: 30 % v/v acetonitrile, 2.5×10^{-2} K_2HPO_4, pH 7.5. 30°C.

C_{14}BDAC Concentration ($mol.dm^{-3}$) x 10^4	κ	
	SHM	CCA
0.00	0.00	0.36
1.00	0.29	1.07
2.00	0.57	2.37
3.00	0.59	2.77
4.00	0.67	3.37
5.00	0.74	3.91

Table 3.40. The capacity ratios, κ, of atenolol and chlorthalidone at different mobile phase concentrations of acetonitrile. Stationary phase: ODS Hypersil (Column H). Mobile phase: 2.5 x 10^{-4} mol.dm^{-3} SOS, 0.1% v/v H_2SO_4. Temperature ambient

Solute	κ		
	Acetonitrile concentration (%v/v)		
	16	20	25
Atenolol	1.38	0.56	0.25
Chlorthalidone	9.21	4.96	2.33

Table 3.41. The capacity ratios of atenolol, chlorthalidone and related compounds at different pairing-ion concentrations (SOS). Stationary phase: ODS Hypersil (Column H). Mobile phase: 25% acetonitrile, pH 2.0 (0.1% H_2SO_4). Temperature:ambient.

Solute	κ		
	SOS concentration (mol.dm^{-3})x10^3		
	1.00	2.00	4.00
Atenolol	0.63	1.00	1.33
Chlorthalidone	2.33	2.33	2.33
4-hydroxyphenylacetamide	*	0.17	0.17
4-hydroxyphenylacetic acid	*	0.67	0.67
4-(2'-hydroxy-3'-isopropylamino-propoxy)-phenylacetic acid	*	2.21	2.96
2-(4-chloro-3-sulphamoylbenzoyl)-benzoic acid	*	7.42	7.38

Table 3.42. The capacity ratios of propanolol, bendrofluazide, 1-naphthol and 5-trifluoromethyl-2,4-disulphonamideoaniline at different tetrabutylammonium phosphate concentrations. Stationary phase: ODS Hypersil (Column H). Mobile phase: 40% v/v acetonitrile, 4 x 10^{-3} mol.dm^{-3} SOS, pH 2.0 (0.1% H_2SO_4). Temperature: ambient

Solute	k' Tetrabutylammonium phosphate concentration (mol.dm^{-3}) x 10^3				
	0.00	0.20	0.40	1.00	2.00
Propranolol	4.46	3.38	2.39	1.79	1.44
Bendrofluazide	6.64	6.46	6.24	6.00	5.92
1-naphthol	5.82	5.77	5.58	5.39	5.10
Trifluoromethyl-2,4-disulphonamido-aniline	0.82	1.00	1.00	0.85	0.80

3.2. Linearity of Detector Response with Solute Concentration.

Table 3.43 The relationship between the peak heights and peak height ratios of tryptophan and concentration injected. Internal standard: 4-aminophenol. Stationary phase: Spherisorb S5 ODS (Column A). Mobile phase: methanol:water (1:1), 4 x 10^{-4} $mol.dm^{-3}$ SDDS, pH 2.2 (citrate buffer). Injection volume 5 µl. Temperature: ambient

Tryptophan Concentration $mol.dm^{-3}$ x 10^{5}	($\mu g.\ ml^{-1}$)	peak height tryptophan mm (mean)		Peak height 4-aminophenol mm	Ratio (mean)	
2.46	(5.02)	15.0 14.0	(14.5)	85.5 85.5	0.175 0.164	(0.170)
4.92	(10.0)	25.5 25.0	(25.3)	90.0 85.0	0.283 0.294	(0.288)
7.38	(15.1)	38.0 39.5	(38.8)	85.0 84.5	0.447 0.467	(0.457)
9.83	(20.1)	49.0 51.0	(50.0)	87.5 87.5	0.560 0.583	(0.572)
12.3	(25.1)	64.5 65.5	(65.0)	86.0 86.0	0.750 0.762	(0.756)

Table 3.44. The relationship between the peak height of tryptophan and concentration injected. Conditions as Table 3.43. Injection volume 100µl.

Tryptophan Concentration $mol.dm^{-3}$ x 10^{6}	($\mu g.ml^{-1}$)	Peak height mm	(mean)
2.46	(0.50)	32.0 36.0	(34.0)
4.92	(1.00)	75.0 73.0	(74.0)
7.38	(1.50)	107.0 108.5	(107.8)
9.83	(2.00)	130.0 132.0	(131.0)
12.3	(2.50)	164.0 166.0	(165.0

Table 3.45. The relationship between the peak height and peak height ratio of tryptophan and the spiked concentration in plasma. Internal standared: 4-chloroaniline. Conditions as Table 4.43, Injection volume 5 µl.

Tryptophan Concentration mol.dm^{-3} x 10^{5} (µg.ml^{-1})		Trp Peak height mm	(mean)	Internal standard mm	Ratio	(mean)
2.46	(5.02)	26.0 24.0	(25.0)	147.0 142.0	0.184 0.169	(0.177)
6.15	(12.5)	39.5 40.5	(40.0)	122.0 124.0	0.324 0.327	(0.326)
9.83	(20.1)	64.0 65.0	(64.5)	127.0 129.0	0.504 0.504	(0.504)
12.3	(25.1)	70.0 64.0	(67.0)	120.0 120.0	0.583 0.577	(0.580)

Table 3.46. The relationship between the peak height of tryptophan and the spiked concentration in saliva. Conditions as Table 4.43. Injection volume 100 µl.

Tryptophan Concentration mol.dm^{-3} x 10^{6} (µg.ml^{-1})		Peak height mm	(mean)
1.23	(0.25)	8.5 11.0	(9.8)
2.46	(0.50)	16.0 15.5	(15.8)
4.92	(1.00)	32.0 32.0	(32.0)
9.83	(2.01)	58.0 54.0	(56.0)
12.3	(2.51)	86.5 84.5	(85.5)

Table 3.47. The relationship between the peak heights and peak height ratios of 6-chlorocinnoline-3-carboxylic acid (CCA) and the concentration injected. Internal standard: 0.10 mgm.ml^{-1} benzoic acid. Stationary phase: ODS Hypersil (Column G). Mobile phase: 30% v/v methanol, 5 x 10^{-4} mol.dm^{-3} C_{14}BDAC, pH 7.5. Temperature: 30°C

CCA Concentration mg.ml^{-1}	Peak height CCA mm	(mean)	Peak height BA mm	Ratio	(mean)
0.0021	29.0		122.0	0.238	
	28.0	(28.6)	120.0	0.233	(0.236)
	28.0		119.0	0.235	
	29.5		124.0	0.238	
0.0041	55.0		120.0	0.458	
	58.0	(57.3)	126.5	0.458	(0.456)
	57.5		126.5	0.454	
	58.5		129.0	0.453	
0.0062	90.0		135.0	0.667	
	80.0	(84.9)	120.0	0.667	(0.663)
	87.5		130.5	0.670	
	82.0		126.5	0.648	
0.0082	98.5		107.5	0.916	
	92.0	(100.8)	99.5	0.925	(0.914)
	98.0		108.5	0.903	
	114.5		125.5	0.912	
0.0103	139.5		125.5	1.112	
	138.0	(136.6)	123.5	1.117	(1.115)
	142.0		126.0	1.127	
	127.0		115.0	1.104	

Table 3.48. The relationship between the peak heights and peak height ratios of sodium 4-hydroxymandelate (SHM) and the concentration injected. Internal standard benzoic acid: 0.10 mgm.ml^{-1} benzoic acid (BA). Conditions as Table 3.47

SHM concentration mg.ml^{-1}	Peak height SHM mm	(mean)	Peak height BA mm	Ratio	(Mean)
0.011	22.5		109.0	0.206	
	22.0	(22.38)	108.5	0.203	(0.203)
	22.0		110.5	0.199	
	23.0		114.0	0.202	
0.022	46.0		107.0	0.430	
	46.5	(46.0)	108.0	0.431	(0.429)
	46.0		108.5	0.428	
	45.5		107.0	0.425	
0.044	94.0		110.0	0.855	
	93.0	(92.5)	108.5	0.857	(0.862)
	91.0		105.0	0.866	
	92.0		106.0	0.868	
0.088	175.0		105.0	1.666	
	177.0	(177.1)	106.0	1.670	(1.673)
	179.0		106.5	1.681	
	177.5		106.0	1.675	
0.110	210.0		100.0	2.100	
	213.0	(215.3)	103.5	2.058	(2.065)
	218.0		106.0	2.057	
	220.0		107.5	2.047	

Table 3.49. The relationship between the peak height and the concentration of 2-hydroxyphenylglycine (2-HPG) injected. Stationary phase: ODS Hypersil (Column G). Mobile phase: 0.1% v/v H_2SO_4, 5 x 10^{-4} mol.dm^{-3} SDDS, 20% v/v acetonitrile. Temperature: 30°C.

2-HPG Concentration mgm. ml^{-1}	Peak Heights mm 1	2	mean
0.010	13.0	13.0	13.0
0.020	24.0	22.0	23.0
0.040	53.0	53.0	53.0
0.081	93.0	94.0	93.5
0.101	126.5	124.5	125.5

Table 3.50. The relationship between the peak areas of atenolol and chlorthalidone and the concentration injected. Stationary phase: ODS Hypersil (Column H). Mobile phase: 25% v/v acetonitrile, 4 x 10^{-3} mol.dm^{-3} SOS, 0.1% v/v H_2SO_4, pH 2.0. Temperature: ambient

Atenolol			Chlorthalidone		
Concentration mg.ml^{-1}	Peak Area (mean)		Concentration mg.ml^{-1}	Peak Area (mean)	
0.21	10384		0.05	3400	
		(10305)			(3355)
	10226			3310	
0.41	20562		0.10	6737	
		(20445)			(6654)
	20328			6570	
0.83	40974		0.21	13372	
		(40909)			(13368)
	40844			13364	
1.03	51770		0.26	16900	
		(51765)			(16934)
	51760			16968	
1.61	83477		0.41	27500	
		(83438)			(27430)
	83399			27360	
2.06	102643		0.52	33821	
		(102762)			(33802)
	102880			33782	

Table 3.51. The relationship between the peak areas of propranolol and bendrofluazide and the concentration injected. Stationary phase: ODS Hypersil (Column H). Mobile phase: 40% v/v acetonitrile, 0.1% H_2SO_4, 5 x 10^{-3} mol.dm^{-3} tetrabutylammonium phosphate, 4 x 10^{-3} mol. dm^{-3}, SOS. Temperature: ambient.

Bendrofluazide			Propranolol		
Concentration $mg.ml^{-1} x 10^2$	Peak Area	(mean)	Concentration $mg.ml^{-1}$	Peak Area	(mean)
1.03	2180	(2291)	0.32	19548	(19473)
	2401			19398	
2.06	5380	(5365)	0.64	39240	(39264)
	5349			39288	
3.09	8462	(8475)	0.96	59479	(59515)
	8488			59551	
4.13	11375	(11332)	1.28	77836	(77900)
	11288			77964	
5.16	14150	(14181)	1.60	97488	(97470)
	14211			97452	

3.3 The stability of Bendrofluazide in Various Solvents.

Table 3.52. The concentration of 5-trifluoromethyl-2,4-disulphonamido-aniline (free amine) produced with time at different temperatures from solutions of bendrofluazide (2 $mg.ml^{-1}$) in different solvents. Concentration is expressed as a percentage of the initial bendrofluazide concentration

Solvent					
HPLC Mobile phase			Acetonitrile		
Time (h)	Temperature (°C)	Free amine (% w/w)	Time (h)	Temperature (°C)	Free amine (% w/w)
0.5	18-22	0.0	1.0	18-22	0.0
23.0	18-22	0.1	23.0	18-22	0.0
6.5	4	0.0	1.5	4	0.0
24.5	4	0.0	24.8	4	0.0
2.0	37	0.0	3.5	37	0.0
26.0	37	0.1	26.3	37	0.0
Acetonitrile:water (2:3)			Borate buffer (pH 9.6)		
Time (h)	Temperature (°C)	Free amine (% w/w)	Time (h)	Temperature (°C)	Free amine (% w/w)
1.5	18-22	0.2	1.8	18-22	0.5
23.0	18-22	1.6	23.8	18-22	1.9
1.5	4	0.1	5.3	4	0.2
24.8	4	0.3	25.0	4	0.4
4.8	37	3.1	4.8	37	1.9
27.5	37	12.1	27.5	37	8.4

Ethanol		
Time (h)	Temperature (°C)	Free amine (% w/w)
2.3	18-22	0.0
23.5	18-22	0.0
6.0	4	0.0
25.0	4	0.0
4.2	37	0.2
27.5	37	0.8

SECTION 4. DISCUSSION

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4.1 Micellisation of the Pairing-ions.

Regularly structured micelles are formed spontaneously in aqueous solutions of surface active agents (97). Micellisation in aqueous solution is a manifestation of the hydrophobic effect (209) and is entropy dominated due to the loss of the highly structured water molecules which are arranged around the alkyl chains of the surfactant monomers (nb. see also ref. 96).

Micelles change not only the physical properties of surfactant solutions but also the solution behaviour of added solutes ions which can distribute into the micellar structure (209). Hence the chromatographic behaviour of solutes in reversed phase systems will be influenced by both a presence of micelles in the mobile phase (120, 165), and by a decrease in free monomer thermodynamic activity. CMC values of surfactants in aqueous solutions are readily available from the literature and may be applied in the rationalisation of chromatographic systems employing surfactant pairing-ions. However in reversed phase ion-pair HPLSC the mobile phases are hydro-organic. The influence of organic solvents such as methanol on the CMCs of surfactants and its consequences in reversed phase HPLSC have been treated qualitatively by Knox and Laird (120).

In this present study the CMC of C_{14}BDAC has been determined in various aqueous methanol compositions by means of conductivity measurements. It can be seen from Table 4.1 that the CMC of C_{14}BDAC decreases with increasing methanol concentration from 2.08×10^{-3} $mol.dm^{-3}$ in water to 1.55×10^{-3} $mol.dm^{-3}$ in 50% v/v methanol in water. Measurements of the CMC at higher methanol concentrations

Table 4.1. The relationship between the critical micelle concentration (CMC) of C_{14}BDAC and the concentration of methanol. 20°C.

Methanol % v/v	CMC mol.dm^{-3} x 10^{3}
0	2.08
10	2.01
20	1.75
40	1.65
50	1.55

were not possible because of the decrease in conductivity due to the methanol being greater than the decrease in conductivity due to micellisation. These results show that literature values of surfactant CMC may be applied with reasonable accuracy to reversed phase ion-pair systems employing hydro-organic mobile phases. Tables 4.2 and 4.3 show that sub-micellar surfactant concentration have been used in this study, although this does not preclude the possibility of the presence of smaller surfactant aggregates (209).

Table 4.2. Literature values for the critical micelle concentration, CMC, of the ABDAC pairing-ions.

ABDAC	Homologue number	Log CMC* mol.dm^{-3}
C_8BDAC	8	-0.514
C_{10}BDAC	10	-1.228
C_{11}BDAC	11	-1.575
C_{12}BDAC	12	-1.921
C_{13}BDAC	13	-2.313
C_{14}BDAC	14	-2.703
C_{16}BDAC	16	-3.342

Table 4.3. Literature values for the critical micelle concentration, CMC of the alkylsulphate pairing-ions.

Alkylsulphate	Homologue number	Log CMC a mol.dm^{-3}
SOS	8	-0.67
SDS	10	-1.37
SDBS	12	-2.07*
STDS	14	-2.77

* Ref. 99.

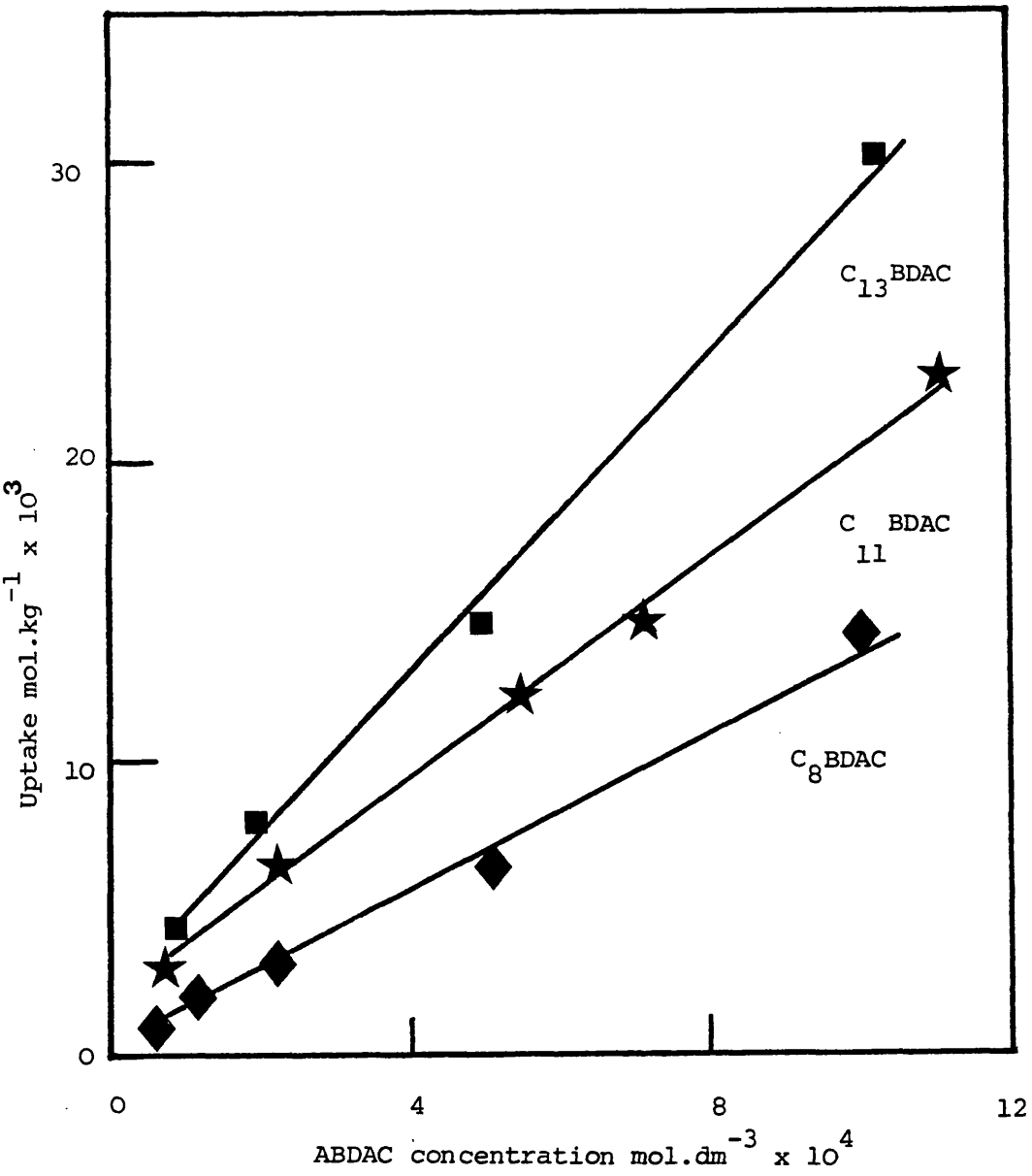
(a) calculated from the value for SDDS using a value of 0.35 (99) for the contribution of each methylene unit to the logarithm of the CMC.

4.2 Adsorption of Alkylbenzyldimethylammonium Chlorides by the Reversed Phase Packing Material.

The adsorption of the C_8, C_{11} and C_{13} homologues of ABDAC onto a reversed phase column packed with Spherisorb S5 ODS was investigated. Figure 4.1 shows that adsorption of the surfactants follows a linear isotherm over the mobile phase concentrations examined and that adsorption increases with increasing homologue number. Uptake of the cationic surfactant onto the stationary phase will result in a positive charge on the surface and the charge density at the surface will depend on both the concentration and alkyl chain length of the pairing-ion in the mobile phase.

The highest adsorption of surfactant was 3.02×10^{-5} mol.gm^{-1} for a mobile phase containing 50% v/v methanol and 1.03×10^{-3} C_{13}BDAC. Assuming a surface area of Spherisorb of 220 m^{2}g^{-1} it follows that the charge density at the surface is 1.5×10^{-7} mol.m^{-2} compared with an octadecyl surface coverage of 1.5×10^{-6} mol.m^{-2} (carbon loading 5.9% w/w Table 2.10).

Fig. 4.1. Adsorption of three ABDAC homologues by octadecylsilica. Stationary phase: Spherisorb S5 ODS (Column A). Mobile phase: ABDAC in methanol:water (50% v/v). 20°C.



4.3 Functional Group Behaviour

Some 44 functional group values were obtained from the capacity ratios of a series of benzoic acids, 8-azapurin-6-ones and 1,3,5-s-triazines substituted in various positions, using a Spherisorb S5 ODS stationary phase (Column B). Standard mobile phase conditions were used such that the solutes were fully ionised and paired with a mobile surfactant of opposite charge. A constant mobile phase concentration of methanol (50% v/v) was used since previous non ion-pair studies had shown that functional group values are influenced by the organic modifier composition (184). The capacity ratios of the solutes and the chromatographic conditions are given in Tables 3.1 to 3.3 and the functional groups were calculated using Eq. 13, i.e.

$$\tau = \log (\kappa_j/\kappa_i) \qquad (13)$$

All the group values are correlated well with the bulk phase liquid-liquid distribution hydrophobic group parameter, π, indicating that the physico-chemical phenomena underlying liquid-liquid distribution between bulk liquid phases are also controlling retention in ion-pair liquid chromatography systems. The appropriate correlation equations (analysed by least squares linear regression) are given below:

Benzoic acids

$$\tau = 0.49\pi + 0.03 \quad ; \quad n = 10,\ r = 0.940, \qquad (103)$$

Azapurinones

$$\tau = 0.49\pi - 0.01 \quad ; \quad n = 10,\ r = 0.983, \qquad (104)$$

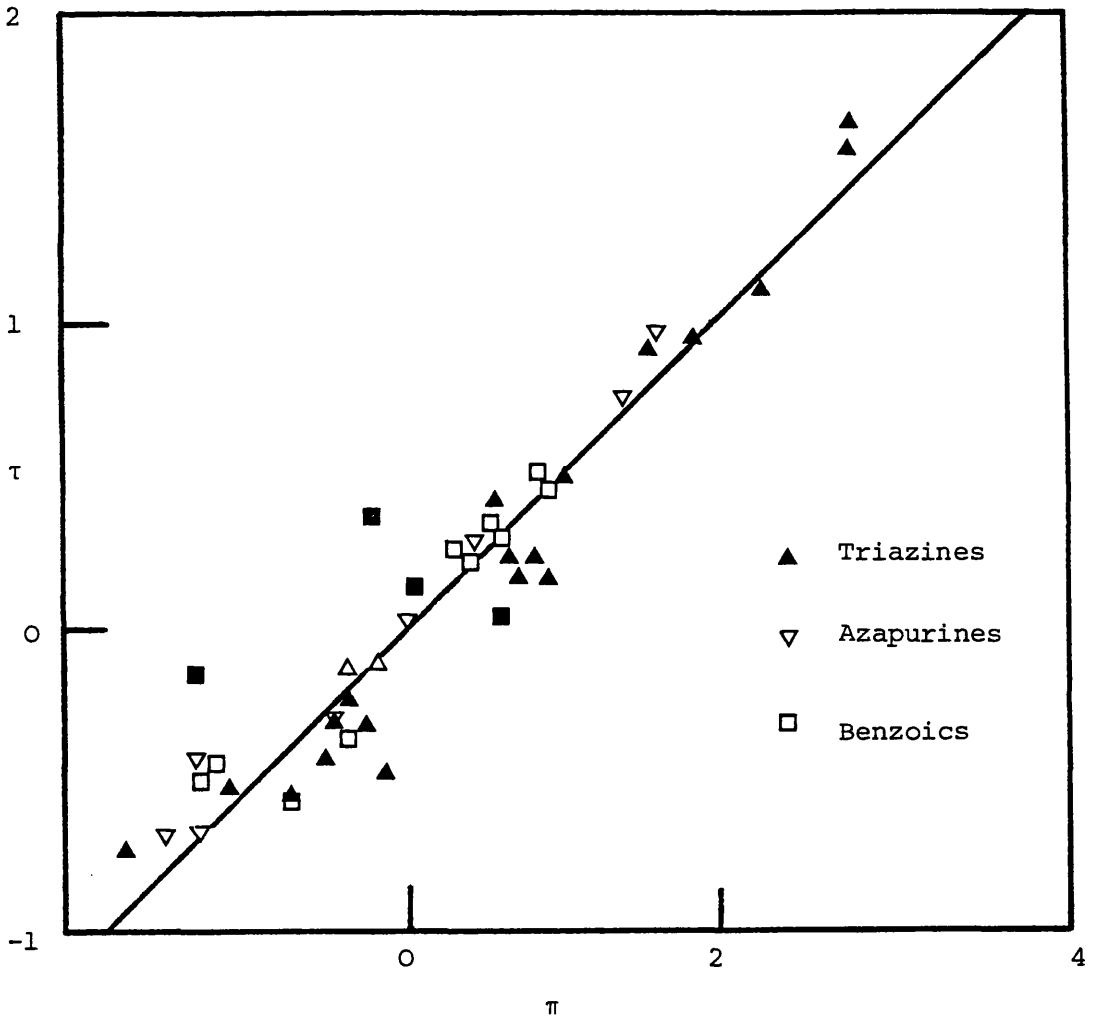
Triazines

$$\tau = 0.51\pi - 0.05 \quad ; \quad n = 20,\ r = 0.961, \qquad (105)$$

All series

$$\tau = 0.49\pi - 0.02 \quad ; \quad n = 40,\ r = 0.965 \qquad (106)$$

Fig. 4.2. Chromatographic group values, τ, *versus* liquid-liquid distribution hydrophobic group values, π, Eq. 107 for three solute series. Chromatographic conditions as Tables 3.1 - 3.3. The closed symbols for the benzoic acids refer to the 2-substituents.



$$\tau = 0.47\pi - 0.002 \quad ; \quad n = 44,\ r = 0.941 \qquad (107)$$

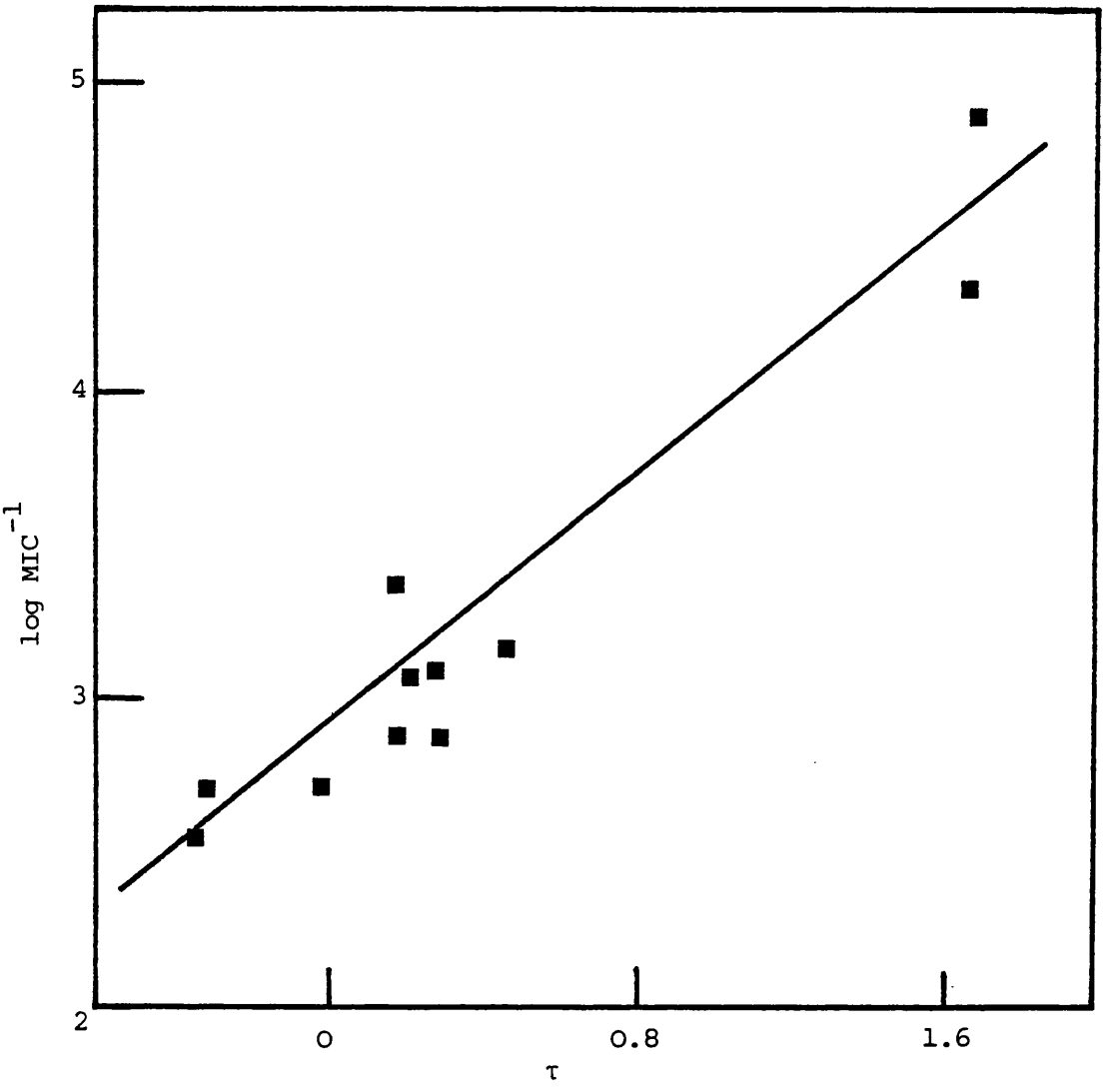
where n and r refer to the number of data points and the correlation coefficient respectively. π values (water-octanol) have been taken from refs. 182, 210 and 211 for the benzoic acid series, refs 182 and 210 for the triazines and ref. 182 for the azapurinones. Equation 103 is for the 3- and 4- substituents, whereas Eq. 107 includes the 2-substituted benzoic acids. The reduction in the correlation coefficient for Eq. 107 compared with Eq. 106 indicates a strong steric contribution of the 2-substituent in the benzoic acid series to the group value. None of the correlation coefficients were improved by the introduction of the Hammett electronic term, σ, indicating a negligible contribution of electronic effects to the group values. The similar slope coefficients indicate a constant effect of mobile phase composition on the group values and the general relationship between τ and π is given in Fig. 4.2. A regressed slope for Eqs.103 to 107 of less than one reflects the presence of methanol in the mobile phase as shown previously by Yamana *et al.* (184).

A strong dependence on solute hydrophobicity is difficult to envisage in a purely ion-exchange system, and the above correlations (Eqs. 103-107) add weight to the conclusions of Horváth *et al.* (162) that a solvophobic effect is primarily responsible for retention in reversed phase ion-pair systems. The two proposed retention models namely ion-pair distribution or ion-exchange are almost certainly an over simplification of the real situation. The chromatographic system cannot be considered as a flat stationary surface with a bulk liquid phase passing over it. In reality a dynamic equilibrium between the adsorbed organic modifier and the

adsorbed pairing-ion and the mobile phase can be envisaged, and the interface between the two phases will constitute a significant proportion of the total void volume within the pores of the stationary phase packing material. There will exist within this interface a concentration gradient of organic modifier and pairing-ion and the interaction between the solute and the pairing-ion may occur either in the bulk mobile phase or within the interface. The results here suggest that a solvophobic effect is primarily responsible for retention which is stabilised by an electrostatic interaction occurring at some distance from the stationary hydrophobic phase. The distance from the stationary phase at which the ion-ion interaction occurs will depend on the nature of the solute and the pairing-ions. Tomlinson *et al* (104) have shown that water reinforced ion-pairs can exist in areas of high dielectric when the total ion-pair carbon number is more than about 20, a condition which is satisfied by the systems described above. Smaller ion-pairs would form at a point within the interface where the dielectric constant is lower than that of the bulk mobile phase in which case the retention mechanism would be indistinguishable in practice from an ion-exchange process. However, the physico-chemical parameters governing retention when ion-pairs form in the interface would still be predominantly solvophobic.

It has been argued (176) that liquid chromatography can provide hydrophobicity parameters for use in quantitative structure-biological activity models. It is found here that τ values determined in an ion-pair RP-HPLSC system using sodium dodecylsulphate as a pairing-ion and methanol as an organic modifer can be used as indices of hydrophobicity for relating the

Fig. 4.3. The relationship between the logarithm of the reciprocal minimum inhibitory concentrations (MIC) of some substituted triazines (ref. 208) and their τ values (Eq. 108). Chromatographic conditions see Table 3.3.



physico-chemical properties of a series of triazines to their minimum inhibitory concentrations (MIC) against *S. aureus* i.e.

$$\text{Log } (\text{MIC})^{-1} = 1.0\pi + 2.9 \quad n=10,\ r=0.965 \qquad (108)$$

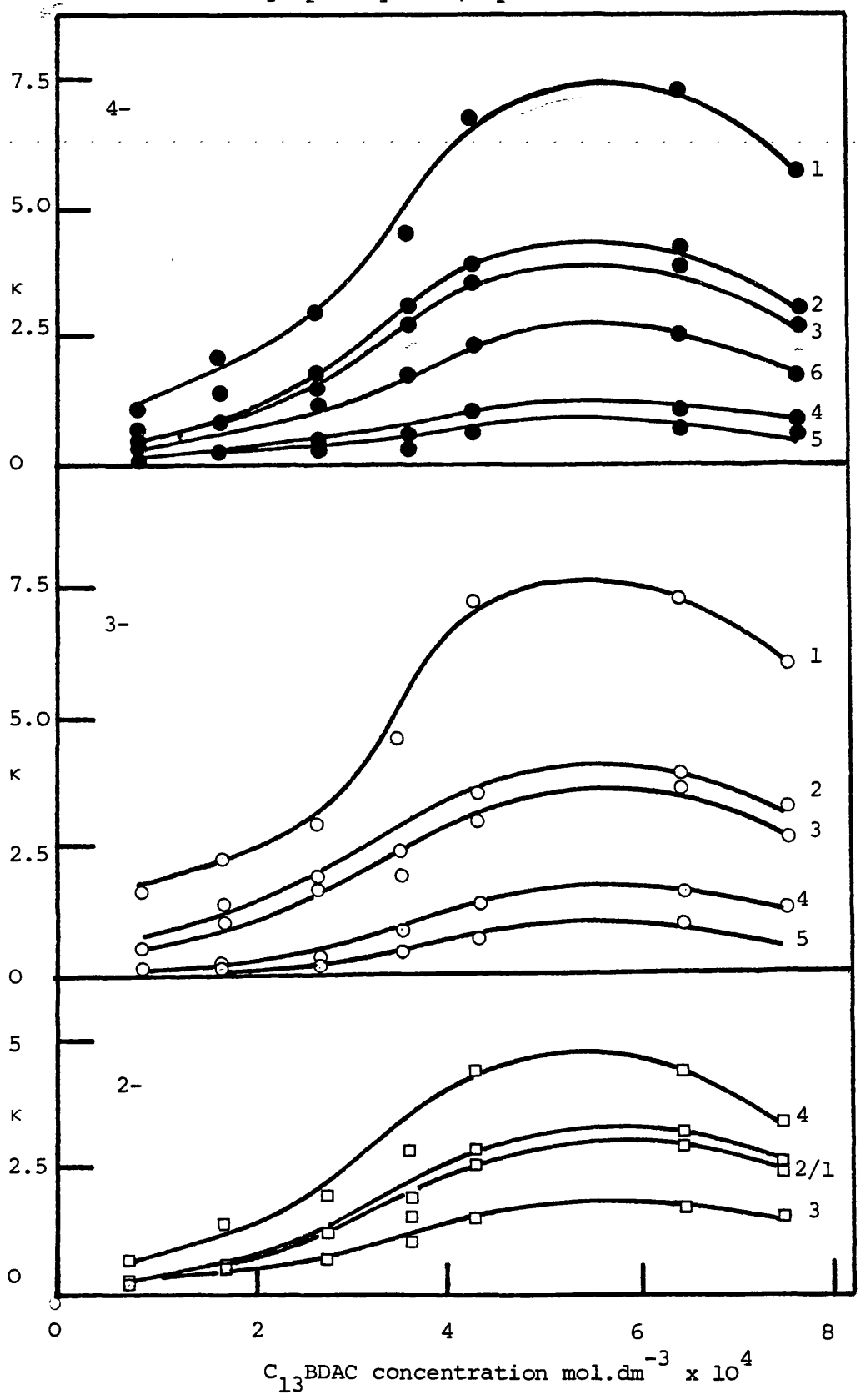
A correlation coefficient of 0.965 for Eq. 108 compares favourably with the value obtained by Wooldridge and co-workers (208) for the correlation of log (1/MIC) with liquid-liquid distribution data. Therefore reversed phase ion-pair HPLSC systems could present a more convenient alternative to partition studies for the prediction of biological activity of antibacterial agents.

4.3.1 Concentration of the pairing-ion

The effect of increasing the mobile phase concentration of C_{13}BDAC over the range 0.93 x 10^{-4} mol.dm^{-3} to 7.48 x 10^{-4} mol.dm^{-3} on the capacity ratios of some substituted benzoic acid derivatives was investigated and the results are shown in Table 3.4 and Fig. 4.4. Figure 4.4 shows that the capacity ratios are quasi-parabolically related to the pairing-ion concentration and hence the data were fitted to the relationship (Eq. 52) derived by Horváth *et al.* The initial sigmoidal effect seen in Fig. 4.4 can be attributed to an initial depletion of pairing-ion at low concentrations such that here retention is related to solute concentration and column load capacity (162). The influence of these side reactions at low pairing-ion concentration is confirmed by the relationship between column efficiency and pairing-ion concentration (Fig. 4.5), in which the plate height reaches a constant minimum at higher pairing concentrations.

Figure 4.6 shows that group contributions towards retention are

Fig. 4.4. Effect of pairing-ion (C_{13}BDAC) concentration on benzoic acid capacity ratios, κ, Table 3.4. Key: 1-5 refer to chloro- methyl, nitro, hydroxy, and amino substituents, respectively, and 6 to the unsubstituted molecules . 2-, 3- and 4-substituents are denoted by open squares, open circles and closed circles, respectively.



independent of pairing-ion concentration in this system above 2×10^{-4} mol.dm^{-3}, indicating that a unique retention process is in operation and that retention can be altered independently of selectivity. Although selectivity is independent of pairing-ion concentration, it follows from Eq. 11 that increasing the pairing-ion concentration will increase resolution due to increases in column efficiency and retention.

The data given in Table 3.4 (omitting κ values obtained below 2×10^{-4} mol.dm^{-3} pairing-ion) was analysed by non-linear regression (using a PDP-11 minicomputer and a standard program employing Marquadt's gradient-expansion method) according to Eq. 52 and the appropriate constants generated are given in Table 4.4. During the analysis the pairing-ion binding constant (K_3) was fixed at 2,750 mol.dm^{-3} based on initial parametization of the 4-chlorobenzoic acid data. Figure 4.7 shows the good linear relationship between log B and π with the *ortho* data points displaced from the *meta* and *para* points, which presumably reflects steric and intramolecular hydrogen bonding factors affecting ion-pair formation, as indicated by their lower ion-pair formation constants (K_2). Since τ has been correlated well with substituent hydrophobicity (Eqs. 103-107) it follows that the ABDAC pairing-ions act as pairing-ions in a manner similar to that described previously for alkylsulphonates and alkylsulphates (162), and that retention is due to ion-pair distribution. Thus B may now be analysed to obtain the ion-pair distribution constants (K_4) which may be described in functional group terms as:

$$\Delta(\text{Log } K_4) = \log (K_{4j} / K_{4i}) \qquad (109)$$

Fig. 4.5. The relationship between the plate height, H, of 4-nitrobenzoic acid and the pairing-ion concentration (C_{13}BDAC). Conditions as Table 3.4.

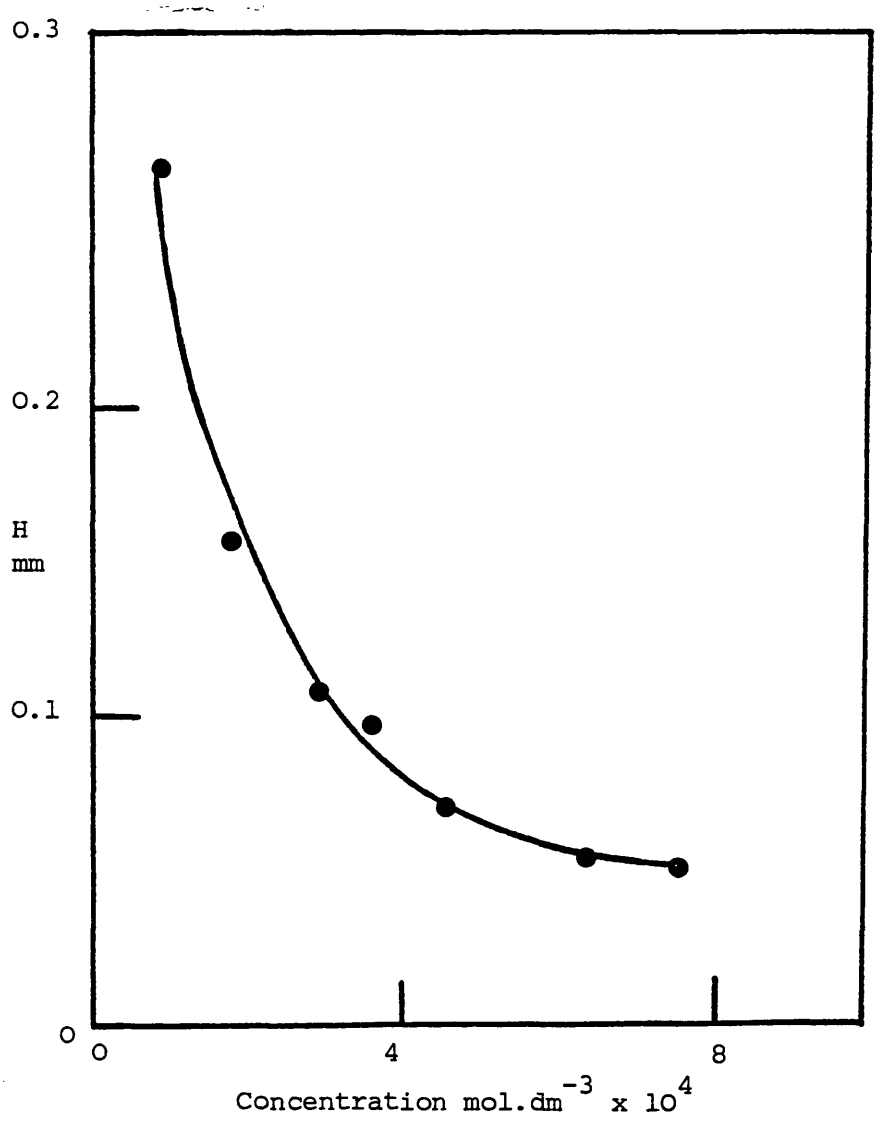
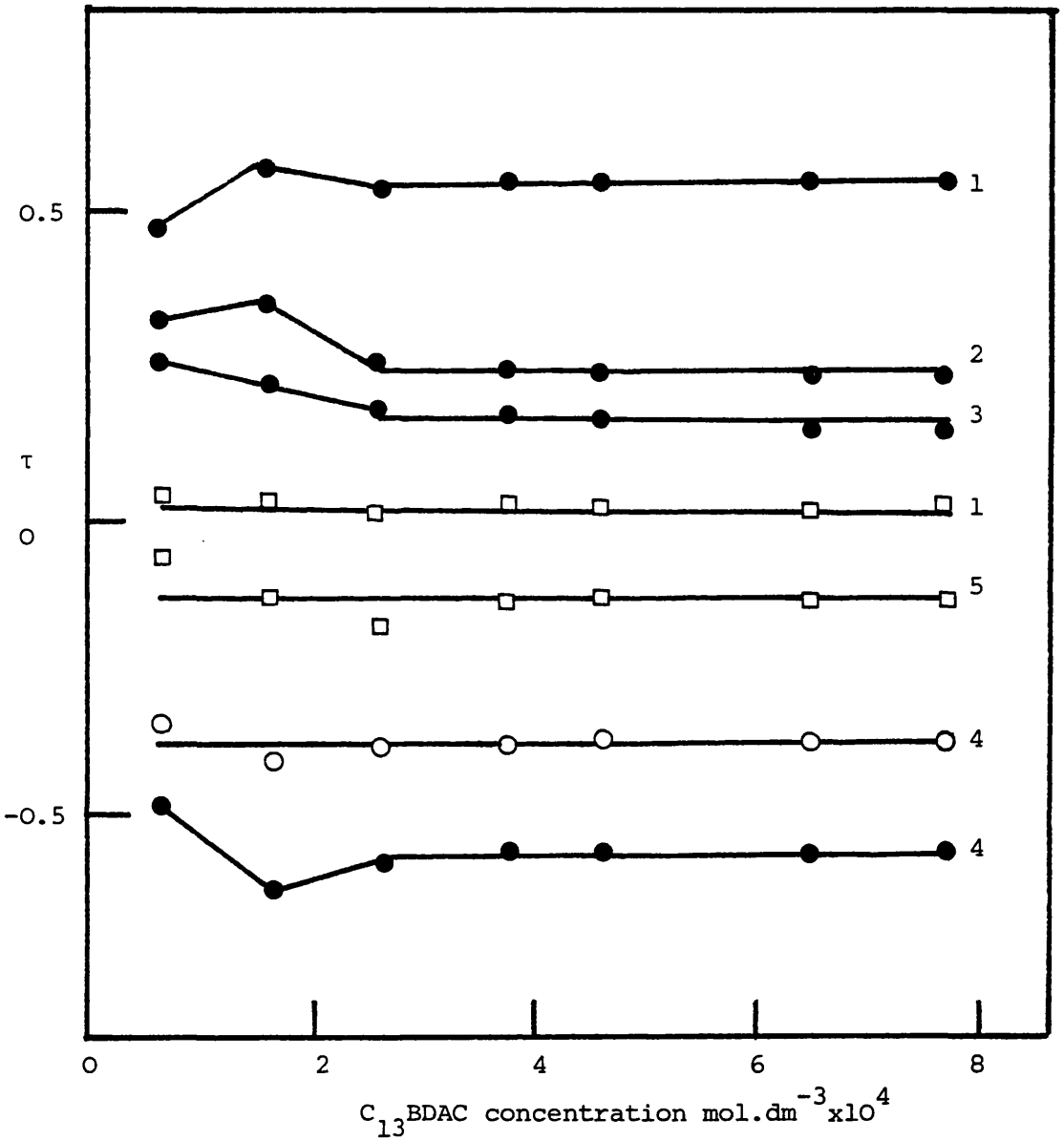


Fig. 4.6. Relationships between pairing-ion concentration and τ for benzoic acids using the same conditions as Fig. 4.4. The plot shows constant τ values above about 2 x 10^{-4} mol.dm^{-3} pairing-ion concentration. Key as Fig. 4.4.



Group chromatographic ion-pair distribution constants, $\Delta\log K_4$, and liquid-liquid distribution constants, π, can be related as shown in Fig. 4.8. Here the π values for benzenoids compiled by Norrington *et al.* (182) have been used to describe functional group bulk phase distributive properties, primarily because of the more complete data set available. Equations 110 and 111 are the appropriate regression equations describing the relationship, i.e.

meta and para

$$\Delta\text{Log } K_4 = 0.43\pi - 0.06 \quad n = 10,\ r = 0.964 \tag{110}$$

$$\Delta\text{Log } K_4 = 0.58\pi - 0.12 \quad n = 8,\ r = 0.948 \tag{111}$$

all substituents

$$\Delta\text{Log } K_4 = 0.32\pi + 0.05 \quad n = 14,\ r = 0.726 \tag{112}$$

$$\Delta\text{Log } K_4 = 0.30\pi + 0.04\sigma + 0.04;\ n = 14,\ R = 0.709 \tag{113}$$

where σ is the Hammett electronic term (168), R is the multiple regression coefficient. Values for Eq. 111 are taken from the compilation of Davis *et al.* (212) and for Eqs. 112 and 113 from ref. 182. Again the inclusion of an electronic term does not improve the correlation indicating that steric effects rather than electronic effects can perturb the general relationship. It is interesting to note that in Fig. 4.8 only the intramolecular hydrogen bonding groups, 2-OH and 2-NH_2 are outliers of the general relationship, compared with Fig. 4.7 in which all the *ortho* points were displaced.

The term B (Eq. 52) may also be given by K_3K_5, where K_3 is the pairing-ion distribution constant and K_5 is the ion-exchange constant. Since K_3 is constant K_5 would be equivalent to B assuming an ion-exchange mechanism to be taking place; again, a dependence of the ion-exchange constant (K_5) on solute hydrophobicity is difficult

Table 4.4. Values of the ion-pair formation constants (K_2), ion-pair distribution constants (K_4) and B ($K_2.K_4$) for the benzoic acids. Conditions as Table 3.4.

Benzoic acid	K_2 (mol.$^{-1}$dm^3)	Standard Error (±)	K_4 mol.$^{-1}$dm^3	Standard Error (±)	B x 10^5 mol.$^{-2}$dm^6
2-HBA	856	116	27.1	3.6	2.32
3-HBA	2,445	306	4.7	0.6	1.15
4-HBA	3,200	504	4.1	0.6	1.31
2-ABA	762	89.4	15.2	2.1	1.16
3-ABA	1,488	153	3.8	0.7	0.57
4-ABA	1,952	206	2.6	0.6	0.51
2-NBA	579	64.8	16.4	1.9	0.95
3-NBA	3,087	392	11.3	1.5	3.49
4-NBA	2,781	317	11.3	1.7	3.14
2-CBA	346	40.6	35.7	4.7	1.24
3-CBA	2,511	324	26.2	4.3	6.58
4-CBA	2,781	413	26.2	4.2	7.29
2-MBA	608	72.5	24.1	3.3	1.47
3-MBA	2,570	317	14.6	2.0	3.75
4-MBA	2,916	402	14.0	1.7	4.08
BA	1,569	199	11.1	1.6	1.74

Fig. 4.7. Log B *versus* τ for benzoic acids. Key as Fig. 4.4.

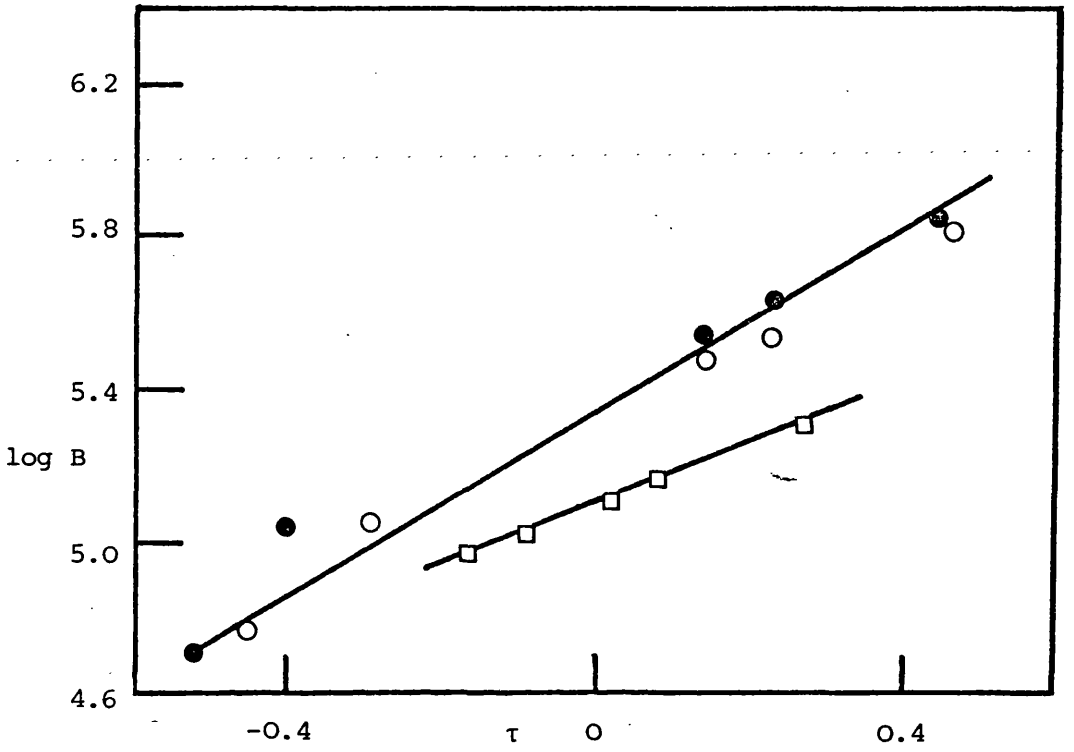
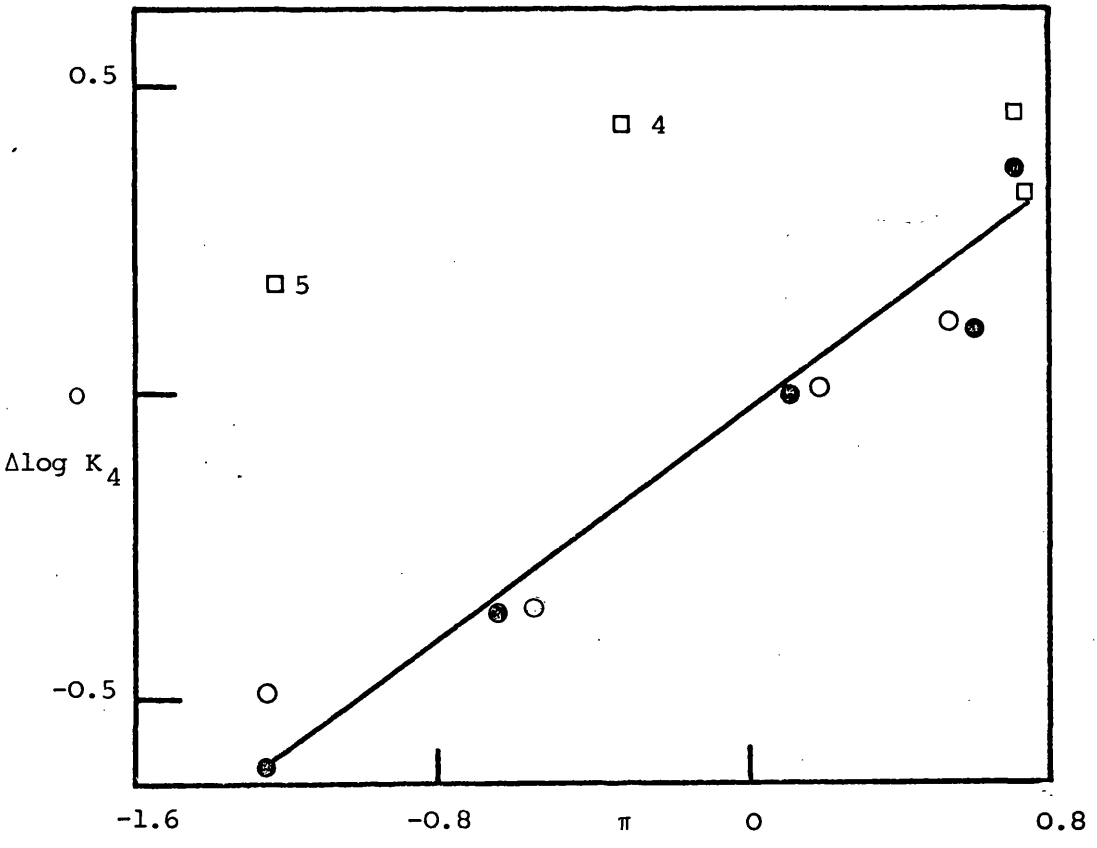


Fig. 4.8. Relationship between chromatographic ion-pair distribution values, $\Delta \log K_4$ and liquid-liquid distribution values, π, for benzoic acids (Eq. 110). Key as Fig. 4.4.



to envisage. This validates the analysis of B in terms of K_2K_4 (cf K_3K_5) and adds more weight to the hypothesis that ion-pair distribution controlled by a solvophobic effect dominates retention in the system under investigation.

4.3.2 The pairing-ion chain length

Since it is demonstrated that the ion-pair distribution constant is related to group values (τ or π), and assuming that it is the total ion-pair which transfers to the stationary phase, it follows that an increase in pairing-ion hydrophobicity although resulting in an increase in retention should not affect functional group selectivity. To test this hypothesis the retention behaviour of a series of benzoic acids was examined using various ABDACs of different chain lengths as pairing-ions. Table 3.6 shows this hypothesis to be correct, i.e. that group selectivity is invariant with pairing-ion hydrophobicity, although retention is not.

The relationship between solute capacity ratio and pairing-ion chain length, n, is given by Eq. 114:

$$\log \kappa = an + b \qquad (114)$$

where a and b are the slope and intercept coefficients respectively. The data was analysed according to Eq. 114 by least squares linear regression and the coefficients are given in Table 4.5 which shows that the slope coefficients for all the benzoic acids can be given by 0.12 ± 0.03 standard deviations. Table 4.5 also shows the regression coefficients according to Eq. 114 for the dianionic carboxylic acid salt sodium cromoglycate and a slope of 0.23 indicates that the methylene group contribution for the pairing-ion with a dianionic solute is twice that of a monoanionic solute.

Table 4.5. Regression coefficients for Eq. 114 (Conditions as Table 3.6).

Solute	a	b	r
3-NBA	0.12	-1.22	0.999
3-MBA	0.12	-1.12	0.999
3-CBA	0.11	-0.78	0.990
BA	0.12	-1.36	0.998
SCG	0.23	-2.12	0.997

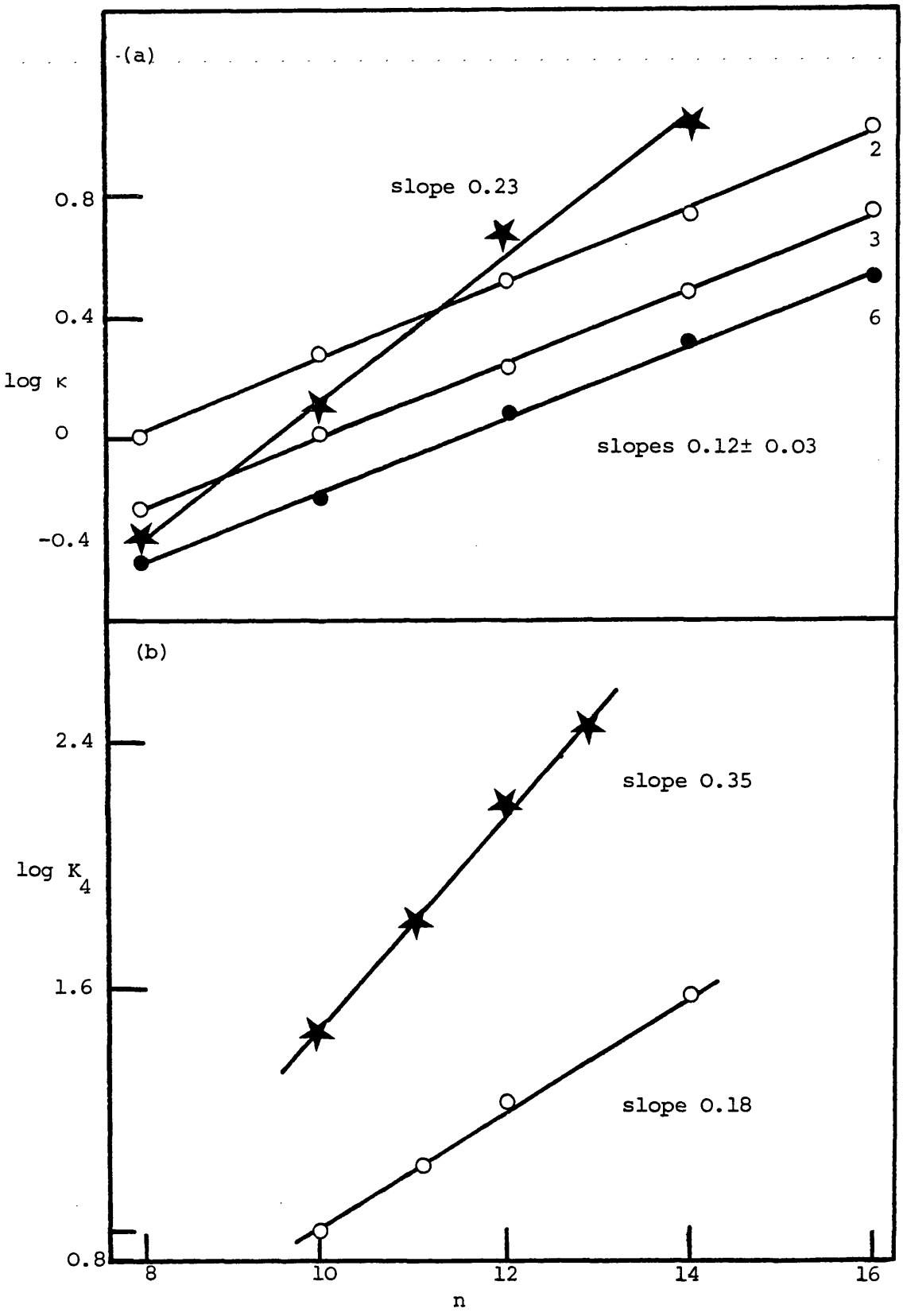
This is indicative of 2:1 stoichiometry for SCG-ABDAC ion-pairs and 1:1 stoichiometry for benzoic acid-ABDAC ion-pairs.

The effect of altering both pairing-ion concentration and alkyl chain length has been examined using 3-nitrobenzoic acid as a model solute and the data compared with that obtained previously for sodium cromoglycate (213). Over the experimentally accessible pairing-ion concentration range (due to ABDAC solubility), and using methanol (40% v/v) as mobile phase a hyperbolic relationship between κ and pairing-ion concentration was obtained for each pairing-ion homologue (Table 3.7). It is interesting to note that a maximum value for capacity ratio was not obtained compared with the present study, (Fig. 4.4), in which a mobile phase of 50% methanol has been employed. This suggests that κ_{max} is determined by the organic solvent composition. The data shown in Fig. 4.4 was analysed according to the hyperbolic relationship given by Eq. 53 to determine the ion-pair formation and ion-pair distribution constants, and these are given in Table 4.6. Figure 4.9b gives plots of K_4 *versus* pairing-ion chain length, n, for the mono- and dianionic solutes. It is seen that, again, the slope coefficient for sodium cromoglycate is twice that for 3-nitro benzoic acid. (Although the slightly different methanol compositions used to obtain the K_4 values would affect the absolute values of K_4 the slopes coefficients would be unaltered). The liquid-liquid distribution coefficients, K_D between water and chloroform for ABDAC-SCG (2:1) ion-association species have recently been determined by Tomlinson and Davis (95), and Eq. 115 shows that these ion-pair bulk phase liquid-liquid distribution coefficients are linearly related to the chromatographic distribution constants.

Table 4.6. The ion-pair formation constants (K_2) and the ion-pair distribution constants (K_4) for 3-nitrobenzoic acid 3-NBA and sodium cromoglycate (SCG) using ABDAC pairing-ions of different chain lengths. Conditions for 3-NBA as Table 3.7, for: SCG 50% methanol in water + ABDAC, temperature 31 °C, stationary phase, Spherisorb S5 ODS (Column A).

Solute	Pairing-ion alkyl chain length									
	10		11		12		13		14	
	K_2 (S.E.)	K_4 (S.E.)	K_2 (S.E.)	K_4 (S.E.)	K_2 (S.E.)	K_4 (S.E.)	K_2 (S.E.)	K_4 (S.E.)	K_2 (S.E.)	K_4 (S.E.)
3-NBA	3244 (108)	6.6 (0.8)	3746 (407)	10.8 (0.4)	3757 (548)	16.4 (0.9)			3654 (352)	35.7 (1.3)
SCG	781 (86)	29.1 (1.3)	822 (92)	64.7 (7.3)	739 (104)	209 (36)	904 (121)	283 (44)		

Fig. 4.9. Relationships between (a) the logarithm capacity ratios, κ, (b) the ion-pair distribution constants, K_4 and the pairing-ion chain length (ABDAC), for the monoanionic benzoic acids and the dianionic sodium cromoglycate . Conditions as Tables 3.6 and 4.6. Key as Fig. 4.4, star symbol refers to SCG.



$$K_4 = 7.35\ K_D + 23.5 \quad n = 3,\ r = 0.999 \qquad (115)$$

The excellent agreement between the two and the slope coefficients of Figs. 4.9 and 4.10 strongly suggest that the retention process can be described as one of ion-pair formation in the mobile phase and distribution to the stationary phase.

Further credence to this conclusion is given by analysis of the ion-pair formation constants (K_2) obtained in this study. With the exception of *ortho* substituted solutes, the K_2 values range from about 1,500 to 3,500 and there is no correlation between the ion-pair formation constants and substituent parameters. It is apparent that the measured ion-pair formation constants are dependent on the magnitude of the solute charge and that the presence of methanol in the mobile phase blurs any dependence on water-structure reinforced ion-pairing as envisaged by Diamond (103). In the absence of any water structuring effects, the ion-pair formation constants for C_{13}BDAC-benzoic acid ion-pairs can be estimated from the data of Tomlinson *et al* . (104) to be of the order of 150 to 300 in pure water. Horváth *et al* have shown the decrease in dielectric upon changing from water to 50% v/v methanol in water increases the ion-pair formation constants by a factor of about ten. Hence the values obtained here for the ion-pair formation constants are consistent with ion-pair formation in the mobile phase. For smaller ion-pairs it is possible that the high bulk mobile phase dielectric precludes ion-pair formation, in which case ion-pair formation will occur at some point closer to the hydrophobic surface, i.e. within the diffusion layer. Scott and Kucera (75) have shown that with aqueous methanol mobile phases the surface of the stationary phase is coated with a monolayer or bilayer of adsorbed methanol. It is

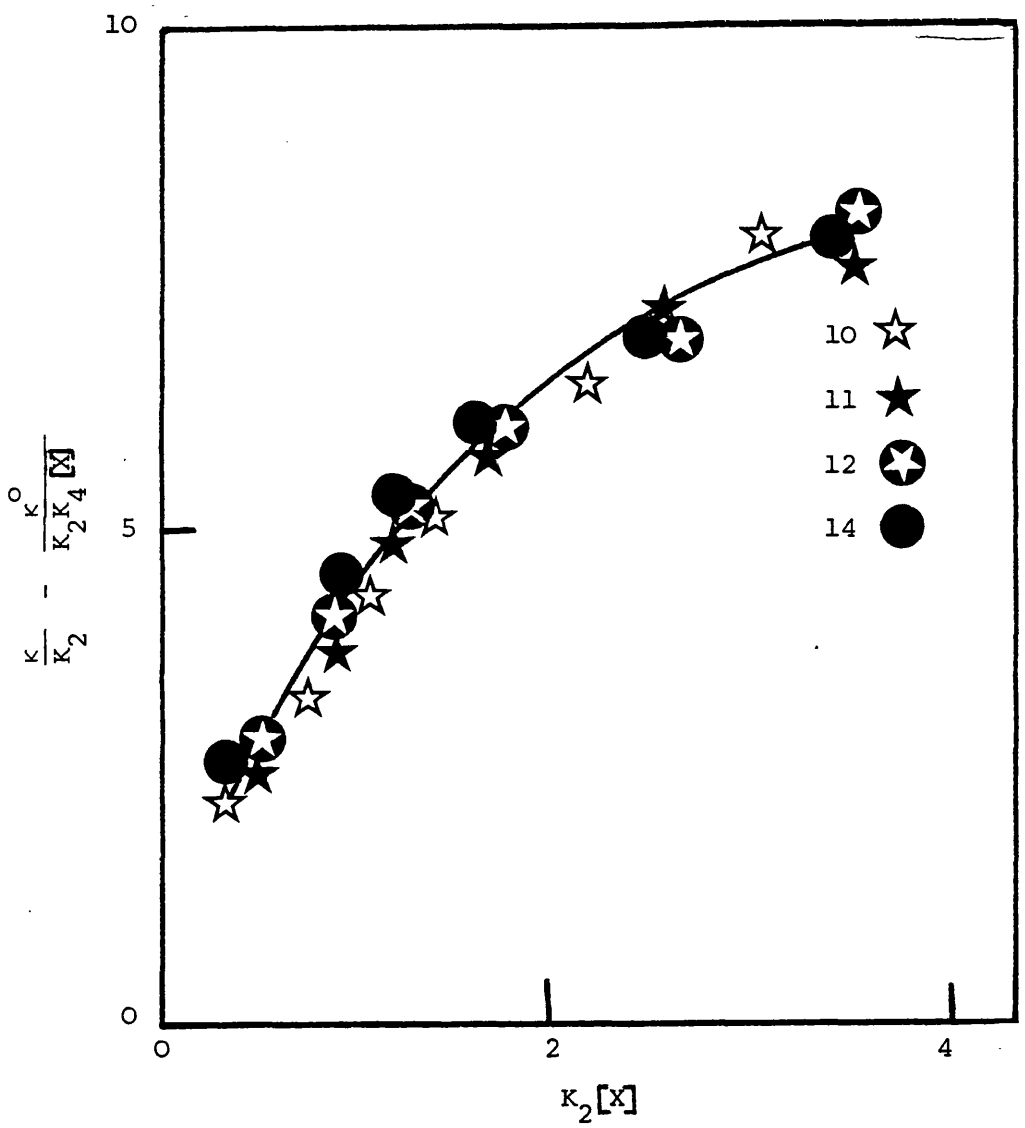
reasonable to assume that the decrease in methanol concentration is exponentially related to the distance from the surface. Akerlof (214) has shown that the solvent dielectric of methanol water mixtures is linearly related to concentration, hence it follows that the solvent dielectric increases exponentially with distance from the surface and is limited by the boundary between the interface and the bulk mobile phase. The position of ion-pair formation is determined by the natures of the solute and the pairing-ion, and the organic solvent composition in the mobile phase. The two extremes will be ion-pair formation in the mobile phase and ion-pair formation at the surface of the stationary phase. However both extremes and all those in between will be controlled by solvophobic forces, and the distribution of such ion-pairs may be discussed within the context of solvophobic theory.

The assumption made in generating K_2 and K_4 from the data in Table 3.7 concerning the omission of low pairing-ion concentrations is seen to be justified by examination of Fig. 4.10 which is a normalised plot of the data fitted by Eq. 53 in terms of K_2, K_4 and the pairing-ion concentration ([X]) describing a hyperbolic relationship passing through the origin (162) and shows the good quality of fit.

4.3.3 Organic modifiers

Ion-pairs formed in this system although large and hydrophobic still retain some polarity (212), and liquid-liquid distribution of ion-pairs is described better in terms of specific solvate formation rather than regular solution theory. Consequently it is to be expected that changes in organic solvent type and concentration

Fig. 4.10. Plot of normalised capacity ratio (ordinate) *versus* normalised pairing-ion concentration (abscissa). Theoretically the relationship describes a rectangular hyperbola passing through the origin and in this case illustrates the good fit of the data. The numbers refer to the pairing-ion chain length.



will cause a complex change in retention and selectivity. To test this hypothesis, four organic solvents, methanol, tetrahydrofuran, acetonitrile and propan-2-ol were compared at different mobile phase concentrations using an ODS Hypersil stationary phase with a fixed C_{14}BDAC pairing-ion concentration (5×10^{-4} mol.dm^{-3}) at pH 7.5 (0.025 mol.dm^{-3} K_2HPO_4 buffer). Tables 3.8 to 3.11 show that the retention of all the benzoic acid solutes studied decreased with increasing organic modifier concentration. Schoemakers *et al.* (216) have predicted from theoretical models that the capacity ratios of a solute will vary quadratically with the volume fraction, ϕ, of organic modifier in a mobile phase binary mixture, i.e.

$$\log \kappa = A.\phi^2 + B\phi + C \qquad (116)$$

It has also been suggested (184) that solute retention should be described as a linear function of the organic modifier volume fraction according to Eq. 117.

$$\log \kappa = D\phi + E \qquad (117)$$

The data obtained in this present study has been analysed by least squares linear regression according to Eq. 117 and the appropriate coefficients are given in Tables 4.7 and 4.8. The introduction of a quadratic term (Eq. 116) was only statistically significant in the case of 4-aminobenzoic acid when tetrahydrofuran was the organic solvent. The values of D and E are dependent upon the benzoic acid substituent character, with values of D reflecting the effect of the organic modifier type on solute retention. The values D may be taken to be a measure of the average solvation number of the solvent and the formed ion-pair (142), and the highest values of D are thus obtained with organic modifier having the greatest extracting ability for the solute ion-pairs. Thus, based on the values of D for benzoic acid these extracting abilities

Table 4.7. Slope and correlation coefficients for the relationship between the capacity ratios of benzoic acids and the organic modifier composition. (Eq. 117). Conditions as Tables 3.8 to 3.11.

Solute	Organic Modifier							
	Methanol		Propan-2-ol		Acetonitrile		Tetrahydrofuran	
	D	r^2	D	r^2	D	r^2	D	r^2
BA	-4.74	0.999	-10.9	0.989	-7.94	0.988	-9.29	0.960
4-ABA	-3.21	0.999	-6.23	0.989	-4.54	0.996	-6.46	0.873
3-ABA	-3.84	0.996	-8.17	0.996	-5.55	0.989	-7.36	0.949
2-ABA	-5.22	0.998	-11.2	0.999	-8.18	0.981	-9.73	0.968
4-HBA	-4.22	0.983	-9.18	0.999	-5.27	0.983	-7.39	0.963
3-HBA	-5.03	0.988	-11.2	0.998	-7.18	0.987	-7.71	0.975
4-NBA	-5.38	0.996	-12.9	0.998	-8.62	0.991	-15.3	0.991
3-NBA	-5.51	0.995	-13.0	0.997	-9.07	0.980	-17.4	0.994
2-NBA	-4.53	0.994	-10.6	0.995	-7.26	0.987	-8.94	0.965
4-MBA	-5.35	0.994	-12.3	0.993	-9.38	0.994	-14.9	0.990
3-MBA	-5.48	0.993	-12.3	0.993	-9.93	0.990	-14.7	0.979
2-MBA	-4.71	0.989	-10.2	0.991	-7.89	0.993	-8.60	0.962
4-CBA	-6.11	0.996			-10.6	0.993	-13.0	0.991
3-CBA	-6.00	0.996			-10.4	0.990	-13.1	0.989
2-CBA	-4.74	0.994	-10.5	0.995	-8.67	0.999	-9.03	0.966

Table 4.8. Intercept values for Eq. 117. Conditions as Tables 3.8 to 3.11.

Solute	Methanol		Propan-2-ol		Acetonitrile		Tetradhyrofuran	
	E	ΔE	E	ΔE	E	ΔE	E	ΔE
BA	2.94	-	3.39	-	2.80	-	2.96	-
4-ABA	1.43	-1.51	1.43	-1.84	1.24	-1.56	1.44	-1.52
3-ABA	1.84	-1.10	2.10	-1.19	1.67	-1.13	1.92	-1.04
2-ABA	3.10	0.16	3.38	-0.01	2.88	0.08	3.08	0.12
4-HBA	2.20	-0.74	2.45	-0.94	1.58	-1.22	2.12	-0.84
3-HBA	2.82	-0.12	3.20	-0.19	2.34	-0.46	2.93	-0.03
4-NBA	3.44	0.50	4.16	0.67	3.36	0.56	5.19	2.23
3-NBA	3.53	0.59	4.16	0.67	3.48	0.68	5.73	2.87
2-NBA	2.75	-0.19	3.10	-0.29	2.64	-0.16	2.77	-0.19
4-MBA	3.53	0.59	4.05	0.66	3.49	0.69	4.74	0.78
3-MBA	3.63	0.69	4.04	0.65	3.66	0.86	4.67	0.71
2-NBA	3.02	0.06	3.19	-0.20	2.79	-0.01	2.73	-0.23
4-CBA	4.14	1.20			4.10	1.30	4.54	1.60
3-CBA	4.10	1.16			4.08	1.28	4.55	1.61
2-CBA	2.85	-0.09	3.29	-0.10	3.06	0.26	2.85	-0.11

are propan-2-ol (-10.9) > tetrahydrofuran (-9.3) > acetonitrile (-7.9)> methanol (-4.7). Clearly such values could form the basis of an elutropic series for solvents used in reversed phase ion-pair systems.

The effect of solute structure on the values of D and E can be seen using a group contribution approach such that

$$\Delta D = D_j.D_i^{-1} \quad (118)$$

and

$$\Delta E = E_j.E_i^{-1} \quad (119)$$

consequently it follows that

$$\tau = \Delta D.\phi + \Delta E \quad (120)$$

Figures 4.11 and 4.12 show the relationship between the group values (τ) and the organic modifier concentration and it can be seen that deviations from linearity are observed for certain polar functional groups, in particular NO_2 in tetrahydrofuran and 3- and 4-OH in all solvents, in which cases ΔD depends on the concentration of organic solvent. The slope and intercept coefficients may be related to other non-chromatographic group values, the Hansch hydrophobic value, π, and the Hammett electronic term, σ, as given below:

a. The slope coefficient (ΔD)

i. Methanol

$$\Delta D = -1.1\pi - 0.36, \quad n = 10, \; r^2 = 0.850 \quad (121)$$

$$\Delta D = -0.98\pi - 0.56\sigma - 0.22, \; n = 10, \; R^2 = 0.933 \quad (122)$$

ii. Propan-2-ol

$$\Delta D = -2.98\pi - 0.51 \qquad n = 8, \; r^2 = 0.824 \quad (123)$$

$$\Delta D = -2.62\pi - 1.39\sigma - 0.22 \qquad n = 8, \; R^2 = 0.905 \quad (124)$$

iii. Acetonitrile

$$\Delta D = -1.80\pi - 0.68 \qquad n = 10, \; r^2 = 0.653 \quad (125)$$

$$\Delta D = -1.74\pi - 0.28\sigma - 0.63 \qquad n = 10, \; R^2 = 0.658 \quad (126)$$

Fig. 4.11. τ *versus* mobile phase volume fraction, ϕ, of (a) methanol and (b) propan-2-ol. Conditions as Tables 3.8 and 3.9. Key: as Fig. 4.4.

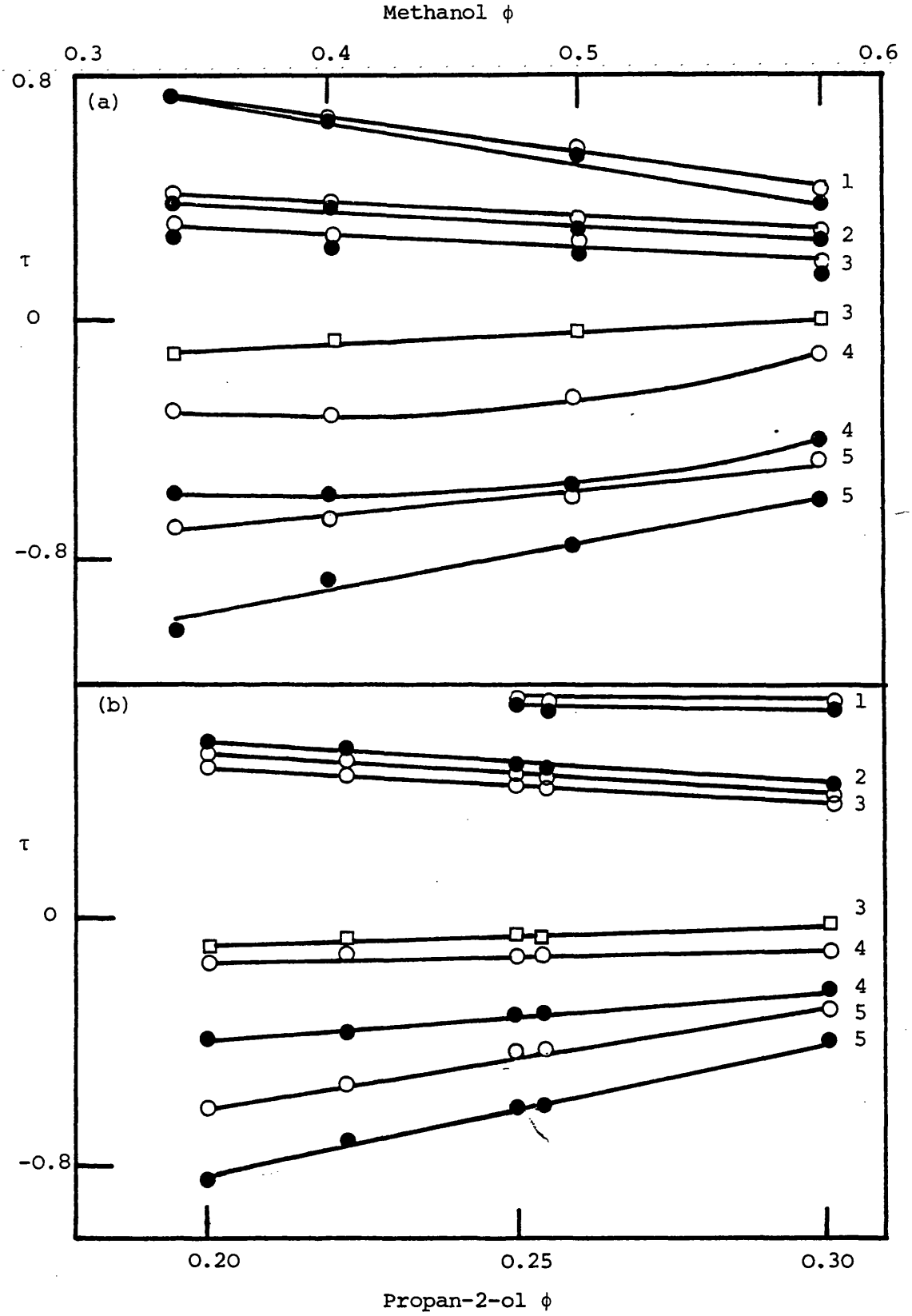
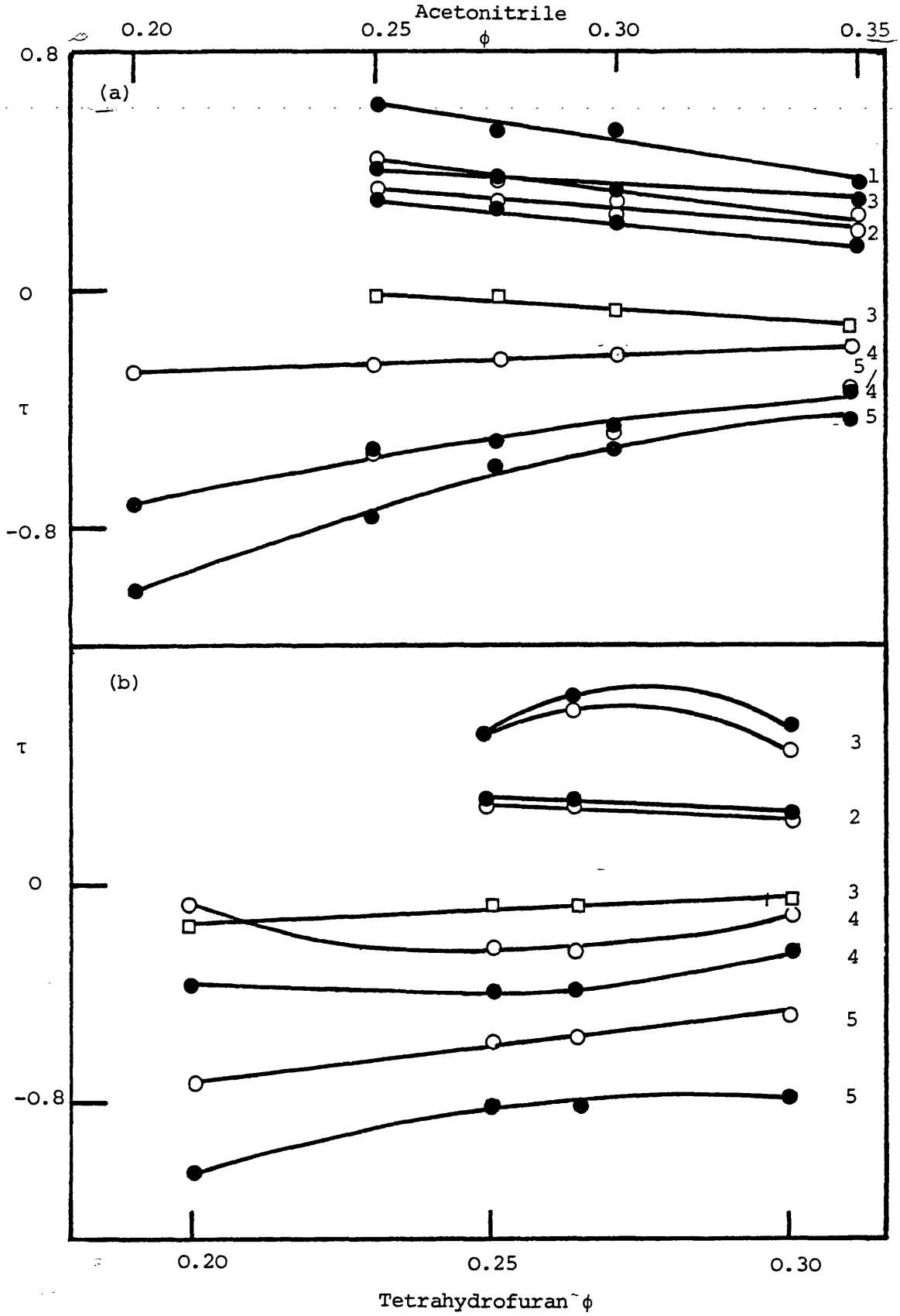


Fig. 4.12. τ *versus* mobile phase volume fraction, ϕ, of (a) acetonitrile and (b) tetrahydrofuran. Conditions as Table 3.10 and 3.11. Key as Fig. 4.4



iv. Tetrahydrofuran

$\Delta D = -4.15\pi - 2.50$ $n = 10$, $r^2 = 0.640$ (127)

$\Delta D = -3.45\pi - 2.83\sigma - 2.17$ $n = 10$, $R^2 = 0.770$ (128)

(The π and σ values are taken from ref 182).

For the two alcoholic organic modifiers it can be seen that the slope coefficient is controlled primarily by the hydrophobicity although there is a significant electronic contribution. For acetonitrile and tetrahydrofuran the dependence of the slope coefficient on substituent hydrophobicity is reduced (i.e. lower correlation coefficients) and it must be assumed to be related to specific solvation effects.

In practical terms the dependence of D on solute hydrophobicity explains the observation of Nice and O'Hare that small changes in organic modifier concentration result in large changes in the retention of very hydrophobic peptides (217).

b. The intercept.

It has been suggested (184) that the intercept values (E and ΔE) may be used as a hydrophobicity index for the calculation of bulk phase partition coefficients (K_D or π). To test this hypothesis in this study the ΔE values were related to π and σ and the found relationships and their significances given by Eq. 129-136.

i. Methanol

$\Delta E = 1.13\pi + 0.23$ $n = 10$, $r^2 = 0.905$ (129)

$\Delta E = 1.04\pi + 0.51\sigma + 0.21$ $n = 10$, $R^2 = 0.980$ (130)

ii. Propan-2-ol

$\Delta E = 1.33\pi + 0.16$ $n = 8$, $r^2 = 0.893$ (131)

$$\Delta E = 1.20\pi + 0.51 + 0.06 \quad n = 8,\ R^2 = 0.952 \qquad (132)$$

iii. Tetrahydrofuran

$$\Delta E = 1.60\pi + 0.86 \quad n = 10,\ r^2 = 0.792 \qquad (133)$$

$$\Delta E = 1.38\pi + 0.14\sigma + 0.27 \quad n = 10,\ R^2 = 0.881 \qquad (134)$$

iv. Acetonitrile

$$\Delta E = 1.32\pi + 0.21 \quad n = 10,\ r^2 = 0.833 \qquad (135)$$

$$\Delta E = 1.22\pi + 0.40\sigma + 0.13 \quad n = 10,\ R^2 = 0.861 \qquad (136)$$

The higher correlation coefficients for methanol and propan-2-ol indicate that they are the more suitable organic solvents for the calculation of intercept terms for hydrophobicity indices,however the correlation of ΔE with π is improved in all cases by the introduction of the electronic term. In theory the values of E and ΔE should be identical for the four organic modifiers since they represent the Log κ and τ values in 100% water. The discrepancies between the values of E and ΔE for the four organic modifiers indicate that the empirical relationships embodied in Eq. 117 should only be used primarily for interpolation, and that extrapolation should be conducted with caution. These discrepancies arise from specific solute-solvent interactions and have been investigated further in this present study by the application of time normalisation approaches (195) and Solvophobic Theory (86-88).

Equation 117 and its usefulness for describing much of the data generated in this present study show that using this relationship it is possible to achieve the required retention for any particular ionised solute at a constant pairing-ion concentration by the appropriate choice of organic modifier.

Organic solvent concentrations can be chosen to give the same retention for a reference compound (in this case benzoic acid) using

methanol (ϕ = 0.50). Thence changes in selectivity (group values) can be shown by relating substituent values obtained using methanol, τ, to those obtained, τ^* in equivalent mobile phases of the other modifiers, i.e.

$$\tau = F.\tau^* + G \qquad (137)$$

Since the retention of the reference solute has been normalised, it is convenient to use the following expression:

$$\log \kappa = F. \log \kappa^* + G \qquad (138)$$

The regression and correlation coefficients obtained for Eq. 138 are shown in Table 4.9 and the relationships are illustrated by the plots shown in Figs. 4.13 and 4.14.

Table 4.9. Regression coefficients for Eq. 138

Organic modifier	F	G	n	r^2
Acetonitrile	1.03	-0.09	15	0.960
Tetrahydrofuran	0.85	0.07	15	0.848
Propan-2-ol	0.99	-0.01	15	0.984

It can be seen that the two alcohols, methanol and propan-2-ol, behave almost identically under time normalisation conditions since all the points lie on a reference line of slope unity. However for the other modifiers the relationships are perturbed by the polar groups NO_2, NH_2 and OH, which are significantly displaced from the reference line of slope unity. It is interesting to note that the hydrophobic substituents CH_3 and Cl show the same selectivity in the four phase systemsas shown by Table 4.10. These results may be explained within the context of the

Table 4.10. Hydrophobic functional group values, τ, in mobile phases containing 'equivalent' organic modifier concentrations. Conditions as Tables 3.8 to 3.11.

Organic modifier		τ CH_3	τ Cl
Type	Concentration ϕ		
Methanol	0.500	0.30	0.53
Propan-2-ol	0.255	0.31	0.54
Acetonitrile	0.275	0.31	0.57
Tetrahydrofuran	0.265	0.28	0.57

Solvophobic Theory. The effect of mobile phase composition on selectivity may be given by Eq. 93 (Section 1.5.3)

$$\tau = -\Delta k_2 + k_3.\Delta(\Delta A) \qquad (93)$$

where Δk_2 is a constant related to the interaction energy of the solvent with the functional group due to van der Waal's forces, $\Delta(\Delta A)$ is the effect of the substituent on the surface area of the solute and k_3 is a term related to the mobile phase surface tension. If the hydrophobic contribution $k_3.\Delta(\Delta A)$, is normalised then differences in mobile phase selectivity are due to different solute-solvent interactions and will be restricted to polar functional groups (see Section 1.5.3).

The perturbations of the relationships embodied in Eq. 138 are more apparent at lower equivalent organic modifier concentrations where the retention of the polar substituented benzoic acids is greater. This effect is demonstrated by Fig. 4.16 which shows the separation of some polar substituted benzoic acids in equivalent mobile phases containing propan-2-ol and tetrahydrofuran. Although

Fig. 4.13. Time normalised retention plots, showing the relationships between (Eq. 166) the capacity ratios of benzoic acids in mobile phases containing methanol (ϕ=0.50) and (a) propan-2-ol (ϕ=0.255) and (b) acetonitrile (ϕ=0.275). The solid line is the reference of slope unity and the hatched line is the regression line (Eq. 138). Key: As Fig. 4.4.

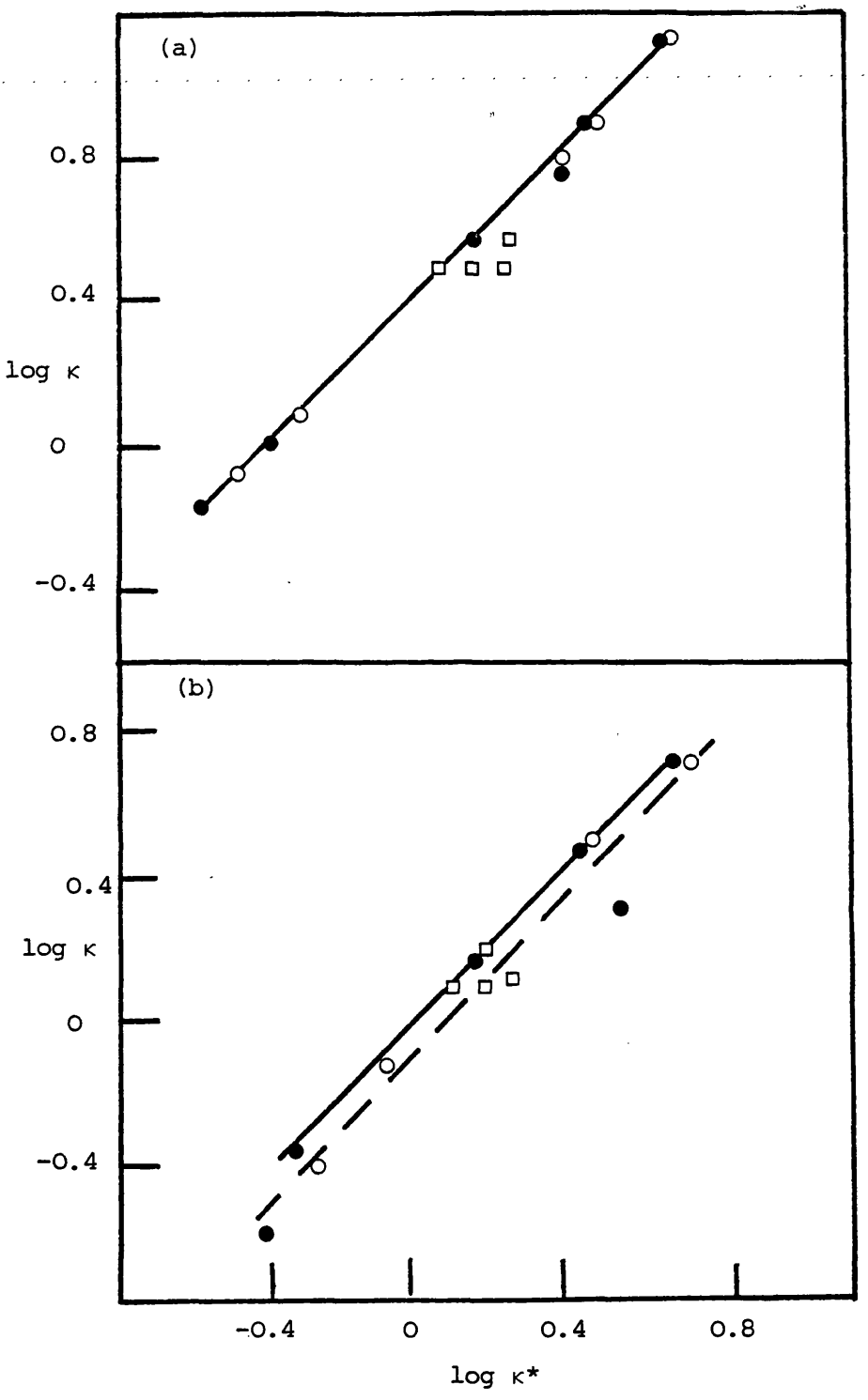


Fig. 4.14. Time normalised plot, showing the relationship between the capacity ratios of benzoic acids in mobile phases containing methanol (ϕ= 0.50) and tetrahydrofuran (ϕ= 0.265). Key as Fig. 4.4.

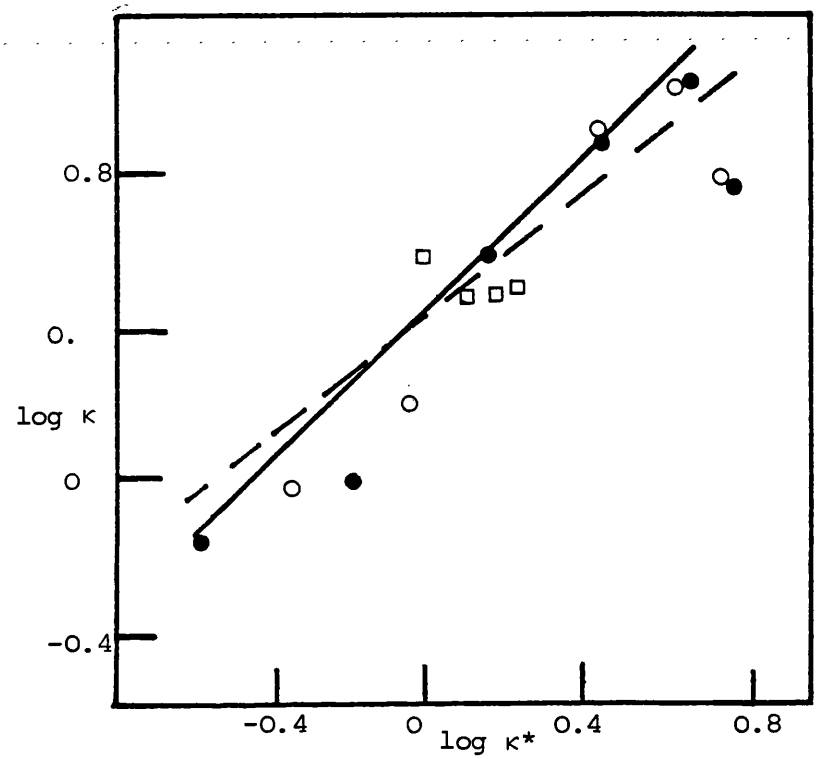
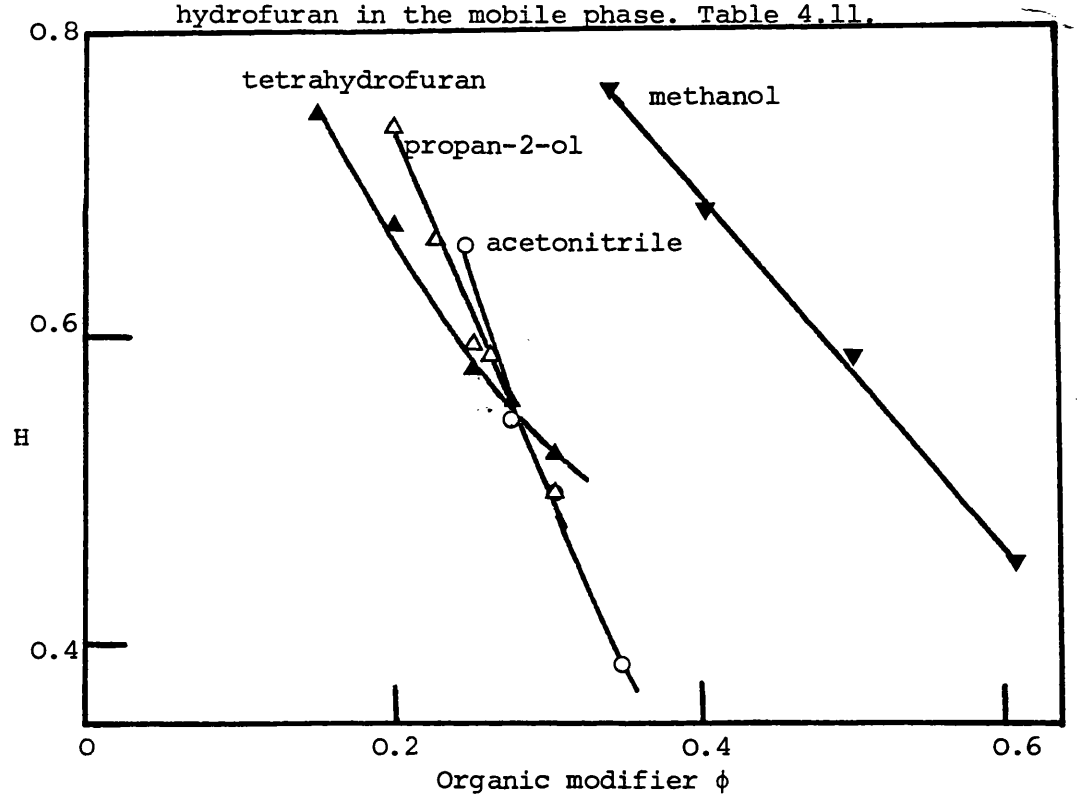


Fig. 4.15. Relationship between the slope coefficient, H,(Eq.139) and the volume fraction,ϕ, of methanol, propan-2-ol,acetonitrile and tetrahydrofuran in the mobile phase. Table 4.11.



these two phase systems produced the same retention for the reference compound, (benzoic acid), significant changes in selectivity and retention order can be observed. These results indicate that if solute polarity in different separations may be improved by changing to an equivalent organic modifier containing a different functional group. Fig. 4.17 shows the equivalent concentrations of the four organic modifiers investigated, and may be used to predict the correct choice of organic modifier for the improvement of separations when solute polarity differences exist (i.e. Δk_2 is significant, Eq. 93).

The effect of organic modifier type and concentration on group selectivity can be demonstrated further by the application of linear free-energy relationships (Section 1.5). Previously in this study, it has been shown that the chromatographic group values are well correlated with the liquid-liquid distribution hydrophobic values, π, (Section 4.3). The data obtained in this section have been analysed according to the general relationship:

$$\tau = H\pi + I \qquad (139)$$

where H and I are the slope and intercept coefficients, respectively. Figure 4.15 shows the relationship between, H and the mobile phase organic modifier composition. The π values used were those described by Norrington *et al.*(182).Figure 4.15 shows that H increases with decreasing organic modifier concentration and that for the two alcohols H is linearly related to the mobile phase volume fraction of organic modifier. For acetonitrile and tetrahydrofuran this relationship is curved and much lower correlation coefficients for Eq. 139 were obtained for these two solvents due to the behaviour of the polar groups described previously. Of particular interest

Fig. 4.16. Separations of some polar benzoic acids, showing the different selectivities of propan-2-ol (a) and tetrahydrofuran (b) at equivalent concentrations. Stationary phase: ODS Hypersil. Mobile phase: 5×10^{-4} mol.dm^{-3} C_{14}BDAC, pH 7.5 (a) 20% v/v propan-2-ol, (b) 20% v/v tetrahydrofuran. 30°C. Key: 1, 4-ABA; 2,3-ABA, 3, 4-HBA; 4, 3-HBA; 5, 2-NBA; 6, 2-ABA; 7, BA.

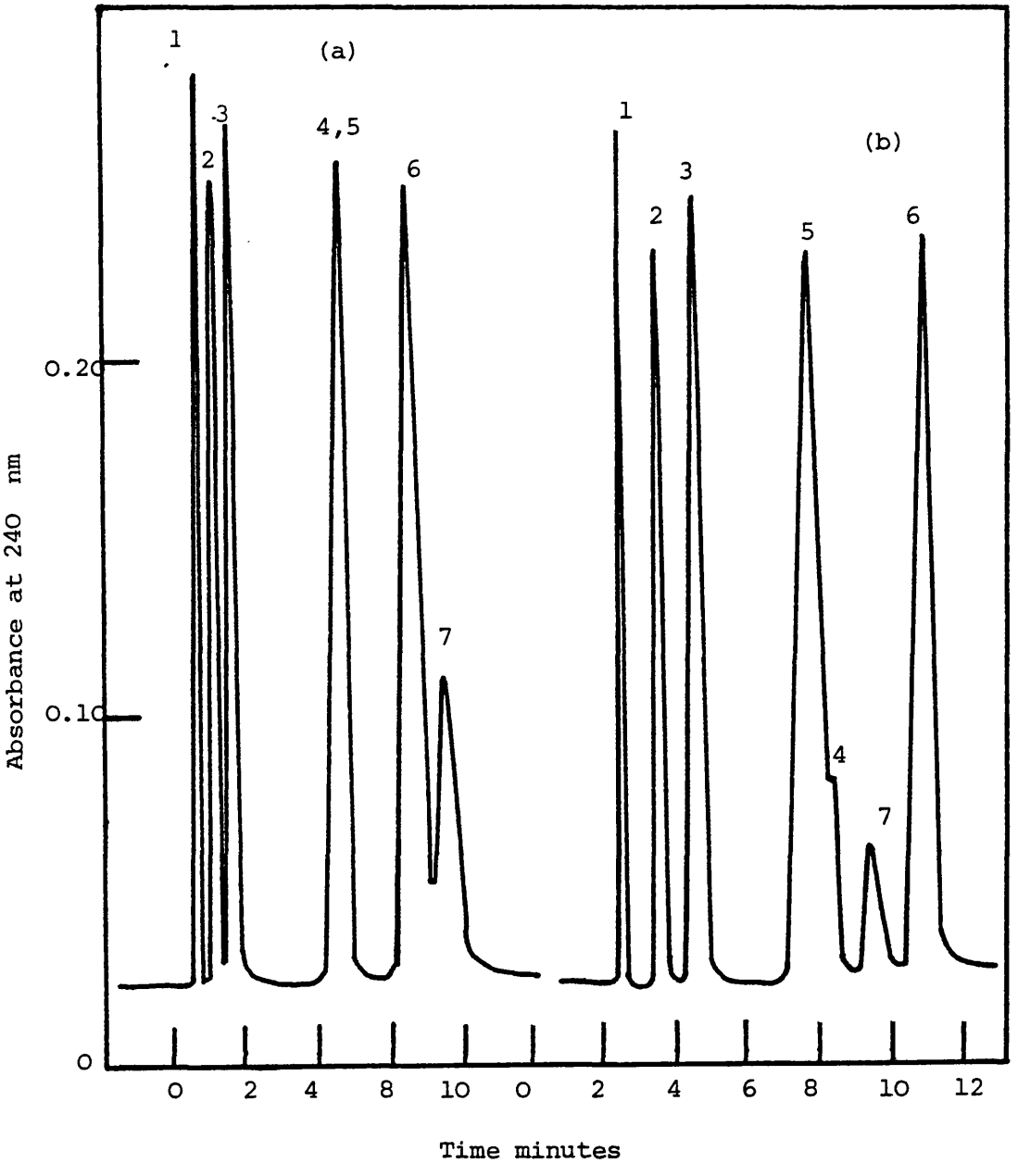


Fig. 4.17. Nomogram showing the equivalent concentrations of four organic modifiers, based on the retention of benzoic acid.

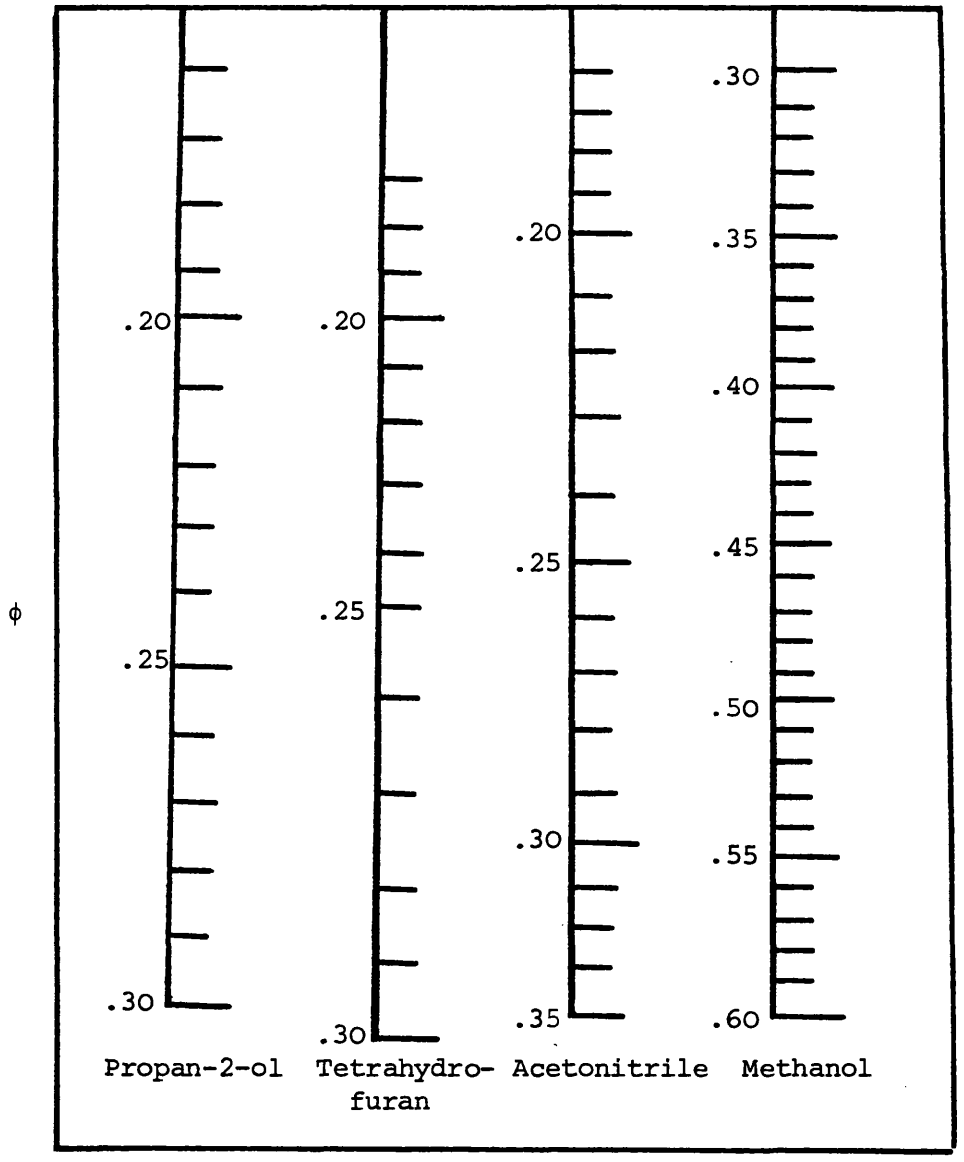


Table 4.11. Eq. 139. Conditions as Tables 3.8 to 3.11.

Modifier Concentration (Volume fraction)	H	I	r	r^2	n
Methanol					
0.34	0.76	0.11	0.978	0.956	11
0.40	0.68	0.09	0.977	0.954	11
0.50	0.59	0.03	0.976	0.953	11
0.60	0.45	0.02	0.983	0.966	11
Propan-2-ol					
0.20	0.74	0.06	0.964	0.930	9
0.225	0.66	0.05	0.968	0.938	9
0.25	0.61	0.04	0.978	0.956	11
*0.255	0.60	0.06	0.977	0.054	11
0.30	0.49	0.05	0.971	0.943	11
Acetonitrile					
0.25	0.66	0.13	0.948	0.899	11
*0.275	0.55	0.09	0.949	0.900	11
0.30	0.49	0.09	0.946	0.895	11
0.35	0.37	0.08	0.926	0.857	11
Tetrahydrofuran					
0.15	0.75	0.07	0.914	0.835	5
0.20	0.67	0.05	0.929	0.863	5
0.25	0.58	0.14	0.901	0.812	11
*0.265	0.56	0.09	0.892	0.796	11
0.30	0.53	0.14	0.915	0.837	11

* equivalent phase systems, based on the retention of benzoic acid.

are the 3- and 4- nitro derivatives which elute before the methyl derivatives when alcoholic mobile phases are employed, (as predicted from their corresponding π values). However when acetonitrile or tetrahydrofuran is employed the elution orders of the nitro and methyl benzoic acids are reversed.

Again, when an organic modifider is present in the mobile phase, the correlation of τ with π was not improved by the introduction of an electronic term, σ. These results indicate that bulk phase partition coefficients may be used to predict retention behaviour in reversed phase ion-pair systems, when alcoholic modifiers are used, however the correlation may be perturbed by specific solvation effects with other modifiers.

The full form of Eq. 93 is given by (218)

$$\tau = \frac{\Delta(\Delta G^{o}_{vdw})_{ji} + N(\Delta A_j - \Delta A_i)\gamma}{2.3\ RT} \qquad (140)$$

where the term $(\Delta A_j - \Delta A_i)$ is a group constant describing the effect of the substituent on altering the surface area of contact of the solute with the stationary phase, and is approximately equal to the surface area of the functional group (192). γ is the surface tension, $\Delta(\Delta G^{o}_{vdw})$ is contribution of the group to the energy of interaction of the solute with the solvent due to van der Waal's interactions, N, R and T are Avogadro's number, the gas constant and the absolute temperature respectively. Hence it is to be expected that functional group behaviour (and retention) is dependent not only on solute-solvent interactions but also on the mobile phase surface tension. To confirm this hypothesis, the capacity ratios of a series of 1,3,5-s-triazines have been determined using

a larger range of organic modifier concentration than previously; i.e. methanol (30 - 96% v/v) and acetonitrile (20 - 96%) (Tables 3.13 and 3.15). The triazine series was chosen because of their known physicochemical properties (208), and the availability of very polar (e.g. SO_2NH_2) and very hydrophobic (e.g. O-n-C_9H_{19}) groups, as well as the fact that their retention behaviour generally permitted a wide range of organic modifier concentration to be examined. The triazine solutes are weak bases, and thus ionised at low pH, consequently an acidic mobile phase (pH 2.2, 0.1% v/v H_2SO_4) containing a constant concentration of SDDS (5×10^{-4} mol. dm^{-3}) pairing-ion was used.

Tables 3.12 and 3.14 and Fig. 4.18a show that over large concentrations of organic modifier the relationship between log κ and organic modifier is non-linear and this effect is particularly noticeable for acetonitrile. Figure 4.19 shows that log κ falls rapidly between 20 and 40% v/v acetonitrile, followed by a plateau between 40 and 70% v/v and a further fall at higher concentrations; this triphasic effect is less pronounced for the more hydrophobic analogues. A comparison of the effects of methanol and acetonitrile is shown in Fig. 4.18a for the retention of the parent, unsubstituted, triazine. This shows that below 70% v/v organic modifier retention is greater in the methanol: water system compared with acetonitrile: water, as indicated by previous data in this section. However above 70% v/v organic modifier the retention order is reversed. Comparison of Fig. 4.18a and Fig. 4.18b (which shows the relationship between organic modifier composition and surface tension), indicates that the dominant factor controlling retention and selectivity is the mobile phase surface tension.

Fig. 4.18. Relationship between organic modifier composition and (a) log capacity ratio, κ, for unsubstituted 1,3,5-s-triazine and (b) mobile phase surface tension, γ. Chromatographic conditions as Tables 3.12 and 3.14. Closed symbols: acetonitrile, open symbols: methanol.

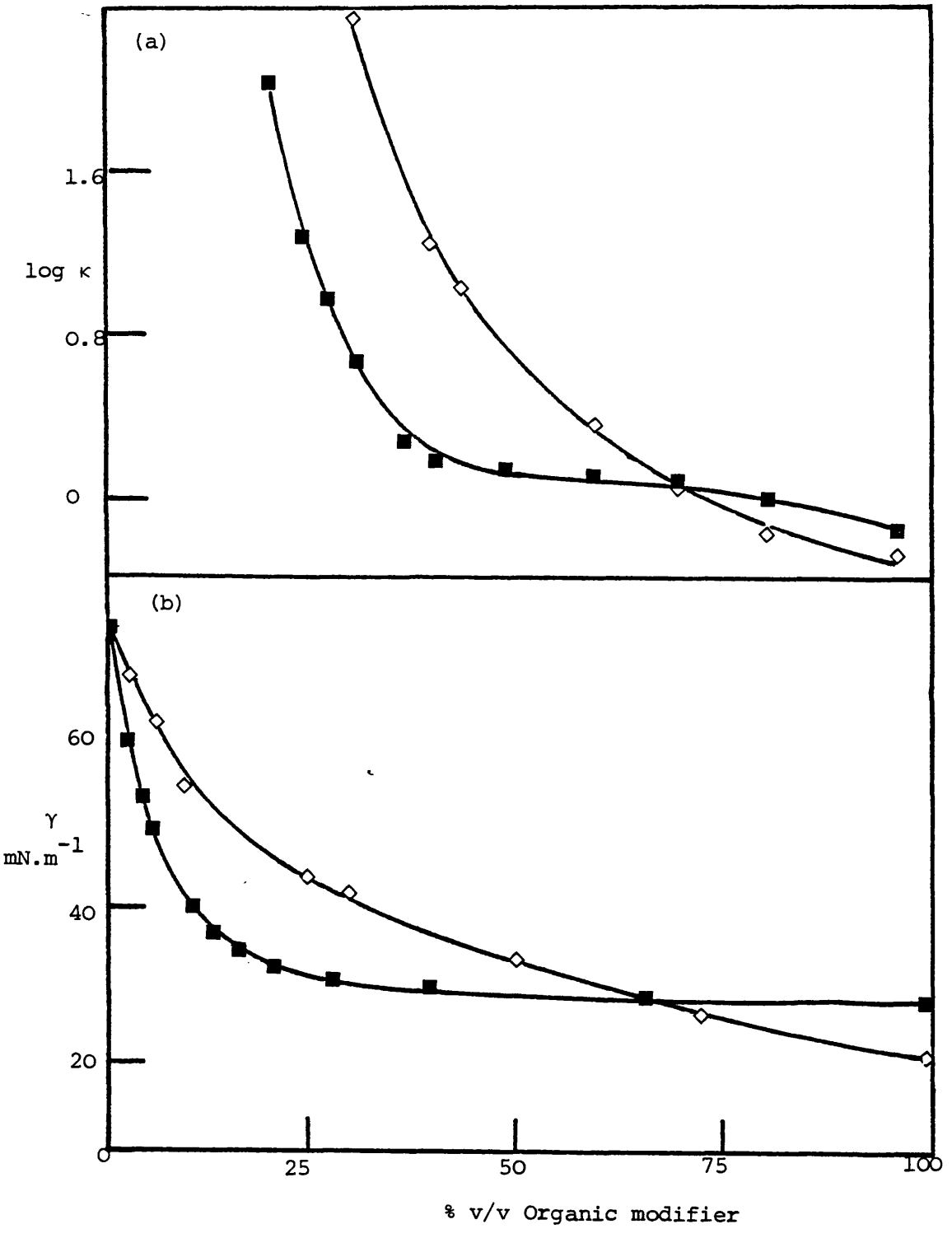
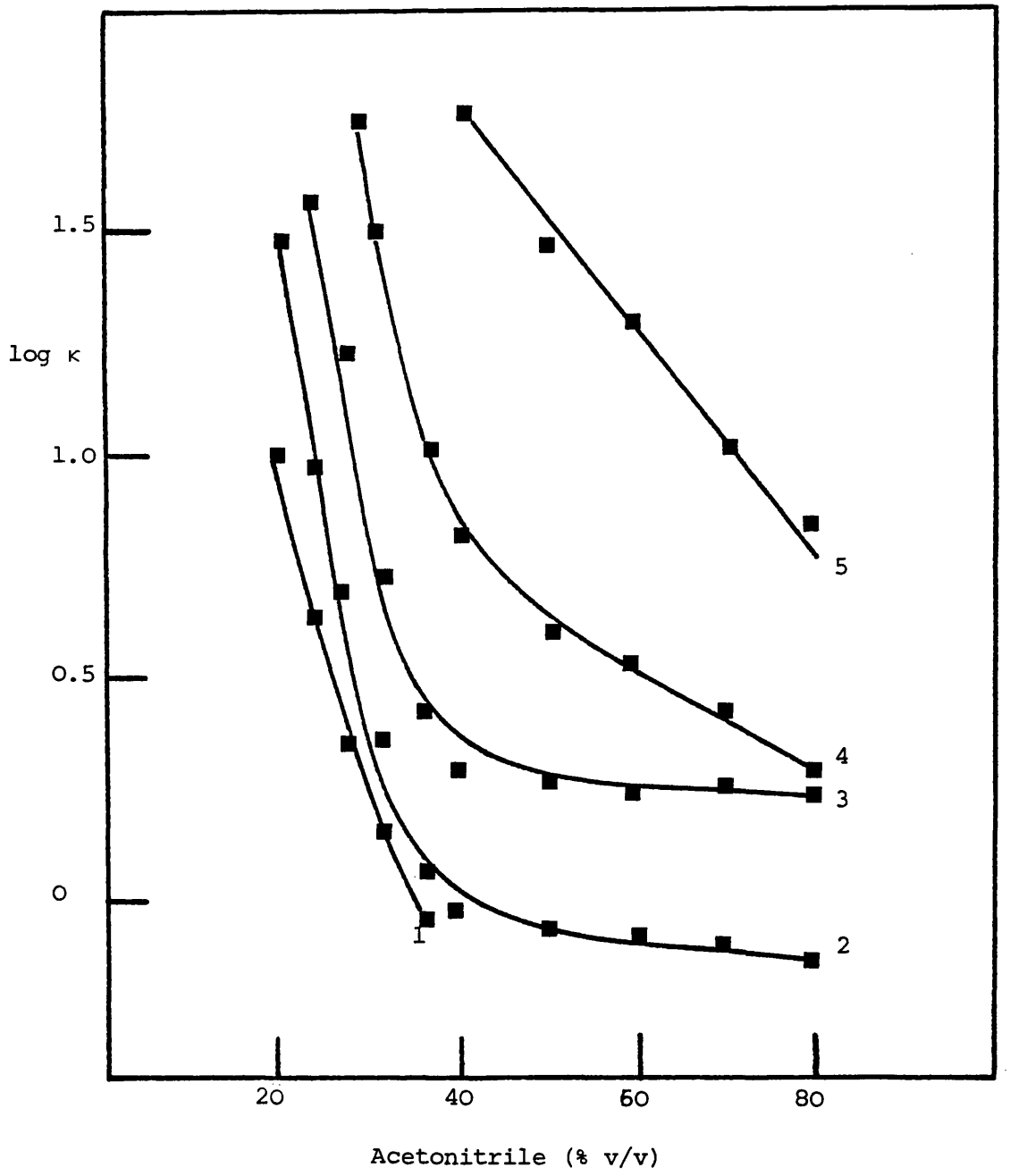


Fig. 4.19. Relationship between log κ and percent acetonitrile mobile phase concentration for some substituted 1,3,5-s-triazines (Table 3.12). 1 - 5 refer to the 3-SO_2NH_2, 4-$NHCOCH_3$, 3-$N(CH_3)_2$ 3-$C(CH_3)_3$ and 3-O-n-C_9H_{19} respectively.



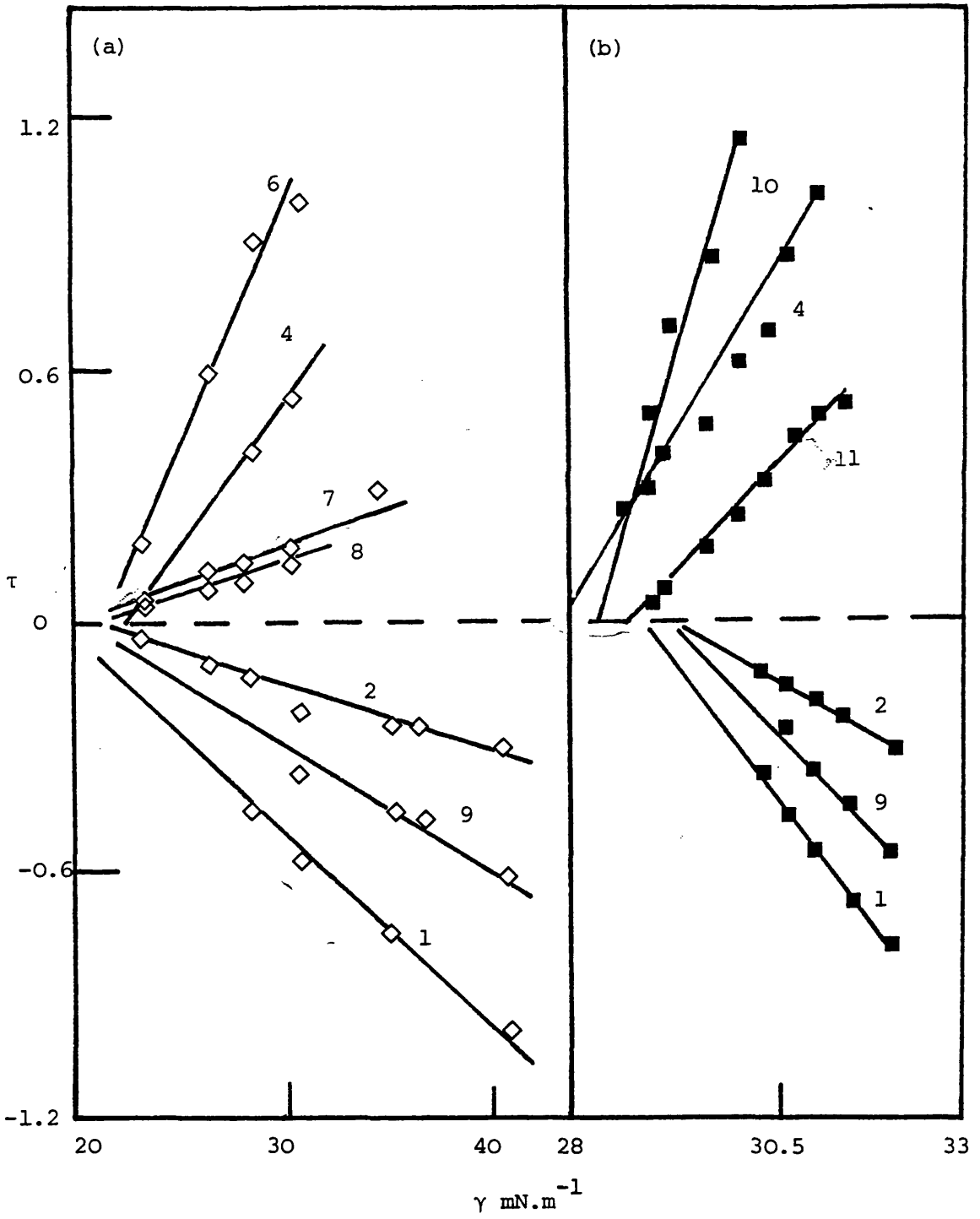
This hypothesis is confirmed by Figs. 4.20a and 4.20b which are plots of group values (Tables 3.13 and 3.15) against literature surface tension. It can be seen that the τ values are linearly related to surface tension and the lines converge at $\tau;\gamma$ 0;21 ($mN.m^{-1}$) and 0.29 ($mN.m^{-1}$) for methanol and acetonitrile, respectively, which correspond to the surface tensions of the pure solvents, which is predicted by Eq. 140.

Slight non-linearity of the τ *versus* γ plots was observed for some of the polar substituents which could be due to the change of $\Delta(\Delta G^{o}_{vdw})$ with changing organic solvent concentration, and the effect of the solute itself on the surface tension. These results suggest that both ion-pair formation and distribution are affected similarly by changes in surface tension. Literature values (219, 220) of surface tension were used in this analysis and the linear relationships between τ and γ show that the effect of buffer salt, injected solute and added surfactant on γ is constant over the organic solvent concentration range studied.

4.3.4 Ionic strength

A priori the results given in the previous Section suggest that any increase in mobile surface tension would increase both retention and group selectivity. An increase in mobile phase ionic strength due to added salt causes a rise in mobile phase surface tension indicating a rise in retention and selectivity. Results presented here and elsewhere (162, 165, 221-223) are to the contrary for ion-pair systems. Figure 4.21 shows the relationship between capacity ratio and mobile phase ionic strength, (controlled by the addition of either KNO_3 or K_2HPO_4), with the pH adjusted to

Fig. 4.20. Relationships between τ and mobile phase surface tension, γ, using (a) methanol and (b) acetonitrile. Chromatographic details see Tables 3.13 and 3.15. Key as Fig. 4.19, and 6 - 11 are 3-O-n-C_6H_{13},3-Br, 3-SCH_3, 3-SO_2CH_3,3-$(CH_2)_4$-phenyl, and 3-CF_3, respectively. Coincidental points have omitted for the purposes of clarity.



7.5 by the dropwise addition of HCl or NaOH. Since the second pKa of phosphoric acid is 6.9 (224) the ionic strength of the phosphate system was calculated by determining the concentration of the two phosphate species using the Henderson-Hasselbalch equation.

The results have been explained empirically by recourse to the presumed retention process that ion-pair distribution dominates. It has been shown (133) that the transfer of ABDAC-cromoglycate ion-pairs from water to chloroform is reduced markedly by the addition of small amounts of NaCl (0.01 to 0.06 $mol.dm^{-3}$), and that this due to a reduction in the association constants between the two ions (104) resulting from shielding of the charge centres (103). If this is the case for the chromatographic systems studied here then τ should be independent of ionic strength, and Fig. 4.22 shows this to be the case.

A comparison of phosphate with nitrate confirms the postulation of charge shielding since Figs.4.21 and 4.22 show that whereas the larger phosphate ions produce a greater decrease in retention compared with nitrate, the functional group values are independent of counter-ion size.

The addition of salt can alter ion-pair equilibria in other ways (225). It can compete with the pairing-ion in forming ion-pairs with the solute ion, or conversely it can form ion-pairs with the pairing-ion so reducing the thermodynamic activity of the latter. These effects are probably reflected in the non-linearity of the κ^{-1} *versus* μ plots found using higher ionic strengths with K_2HPO_4 as the added salts (Fig. 4.21a). The changes in surface tension due

Fig. 4.21. Relationship between reciprocal capacity ratios for benzoic acids and ionic strength, μ, using (a) K_2HPO_4 and (b) KNO_3. Conditions as Tables 3.16 - 3.17. Key as Fig. 4.4.

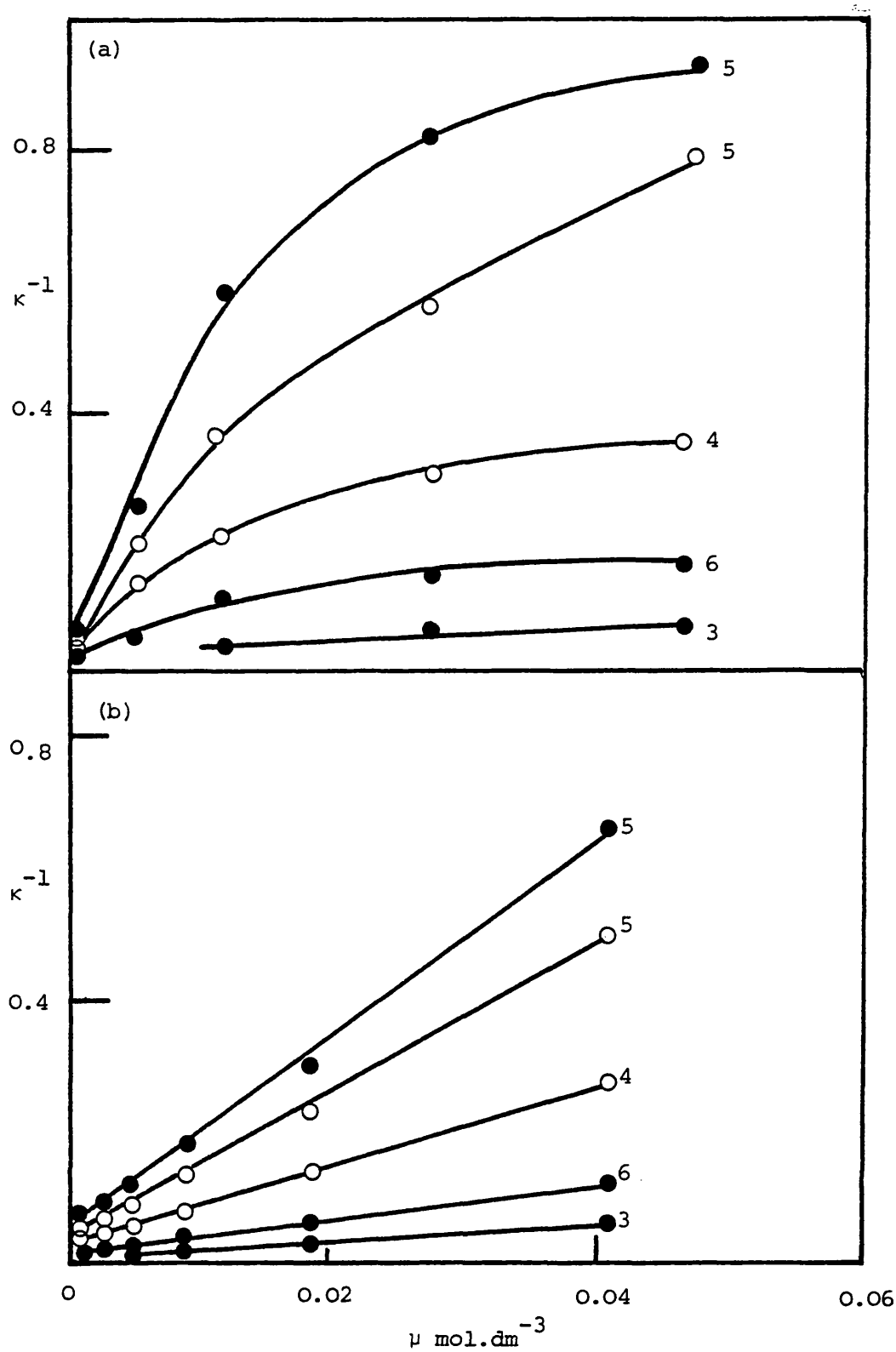
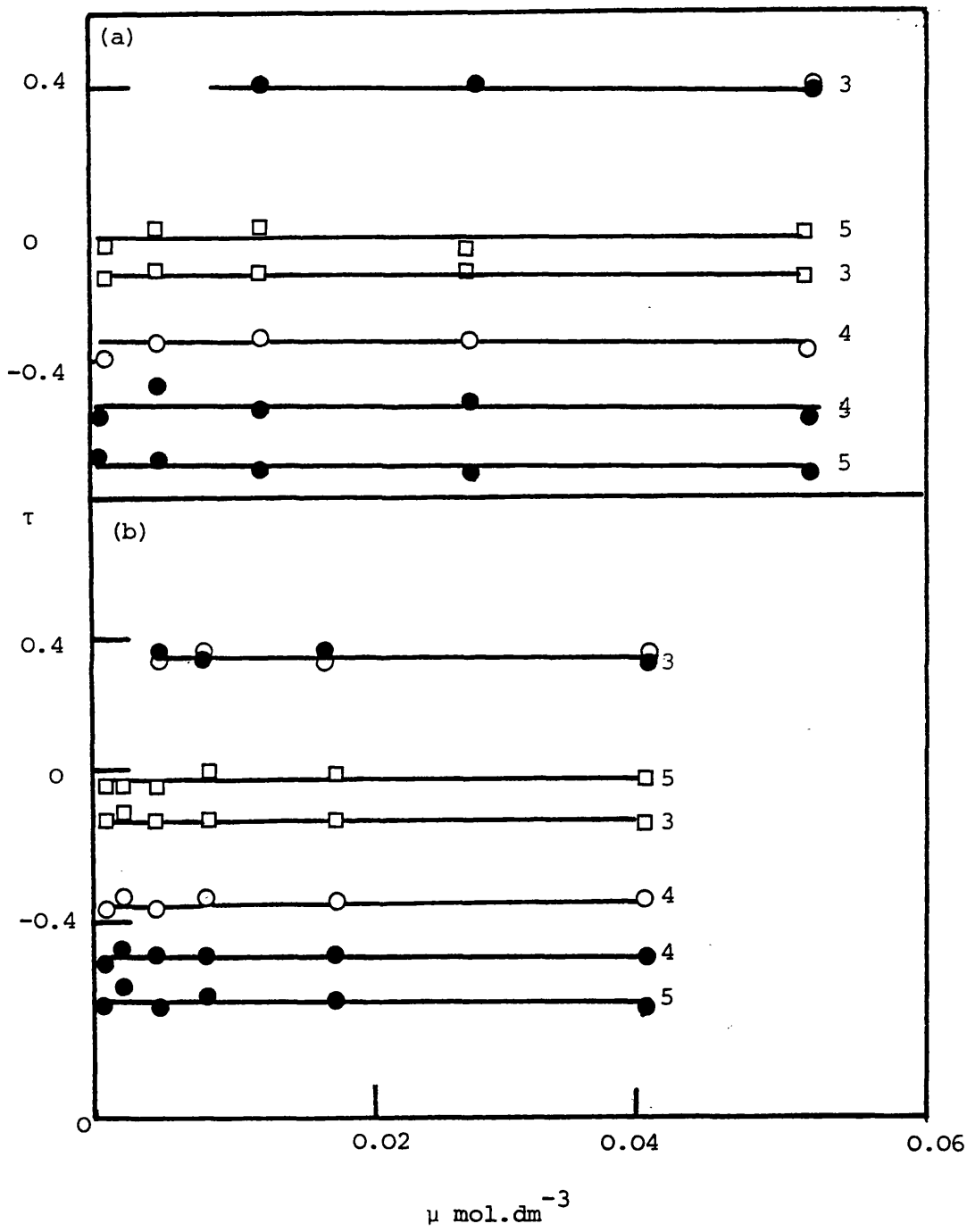


Fig. 4.22. Relationship between τ for benzoic acids and ionic strength, μ, using (a) K_2HPO_4 and (b) KNO_3. Tables 3.18 and 3.19. Key as Fig. 4.3.



to increase in salt concentration were too small over the concentration range studied to have any effect *per se*.

4.3.5 Temperature

Compared with gas chromatography, the effect of temperature in HPLC, and ion-pair systems in particular, has received little attention. The effect of temperature in ion-pair systems is generally reported in qualitative terms (e.g. ref. 226) and there have only been a few instances (165, 200, 227) where detailed studies have been made.

The capacity ratio of a solute is related to the distribution constant of the solute between the mobile and stationary phases, and hence to the standard free energy change (ΔG°), by:

$$\Delta G^{\circ} = -2.3\ R.T.\ \log \kappa - \log V_s.V_m^{-1} \qquad (141)$$

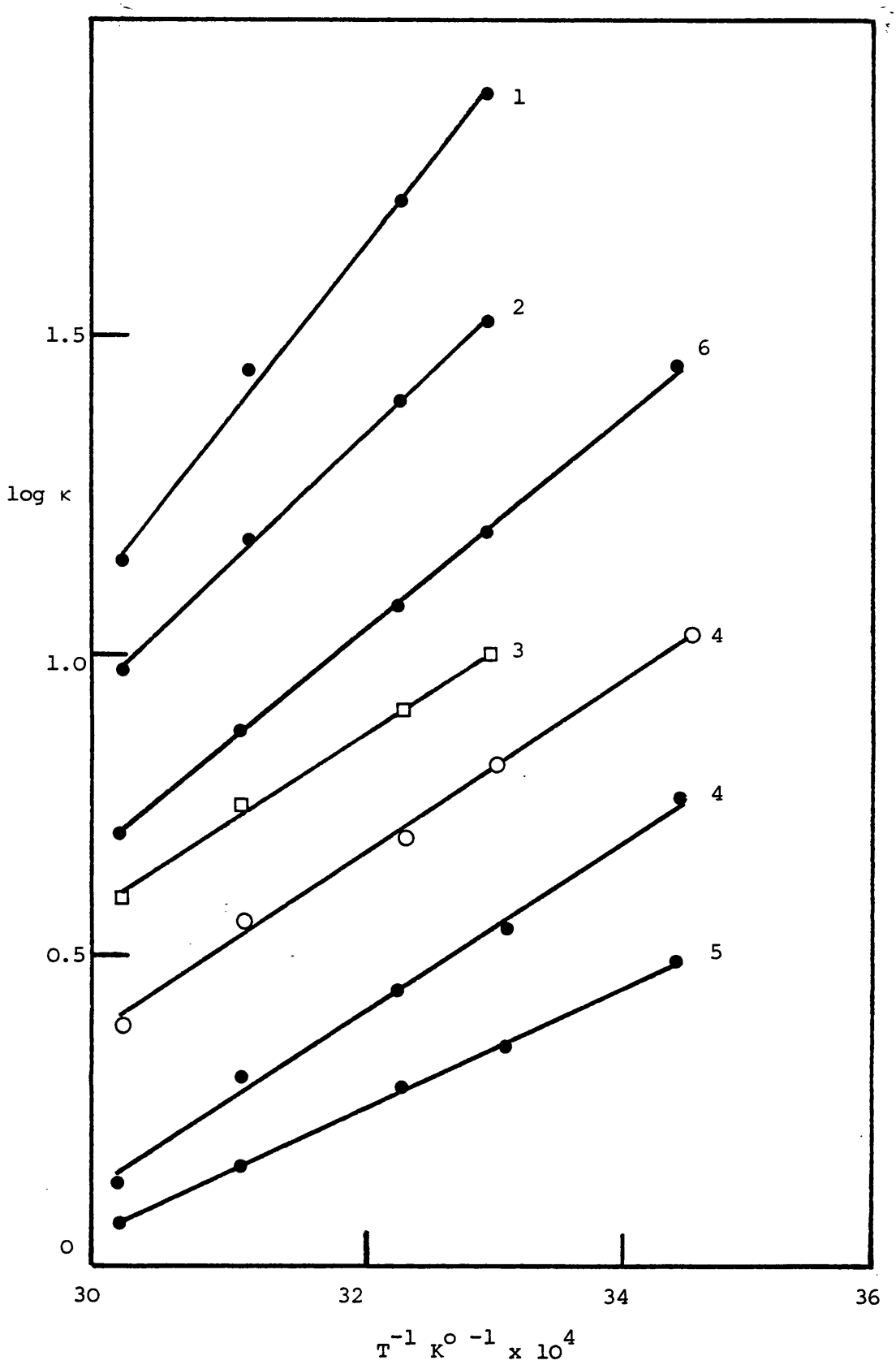
where $V_s.V_m^{-1}$ is the phase volume ratio. By application of the Gibbs equation the capacity ratio may be related to the standard enthalpy and entropy changes, i.e.

$$\log \kappa = \frac{-\Delta H^{\circ}}{2.3R.T.} + \frac{\Delta S^{\circ}}{2.3\ R} + \log V_s.V_m^{-1} \qquad (142)$$

where R and T are the gas constant and the absolute temperatures respectively. Hence the enthalpy change may be determined from a modified Van't Hoff plot of log κ against reciprocal absolute temperature. Due to the difficulty in determining the phase volume ratio in systems using solid bonded phases the entropy change may be only estimated from such plots. In extrathermodynamic terms the functional group value may be related to the temperature as follows (218):

$$\tau = \frac{-\Delta(\Delta H^{\circ})}{2.3\ R.T.} + \frac{\Delta(\Delta S^{\circ})}{2.3\ R} \qquad (143)$$

Fig. 4.23. Van't Hoff plots for benzoic acids, showing the relationship between log κ and reciprocal absolute temperature, T^{-1}. Conditions as Table 3.20. Key as Fig. 4.4.



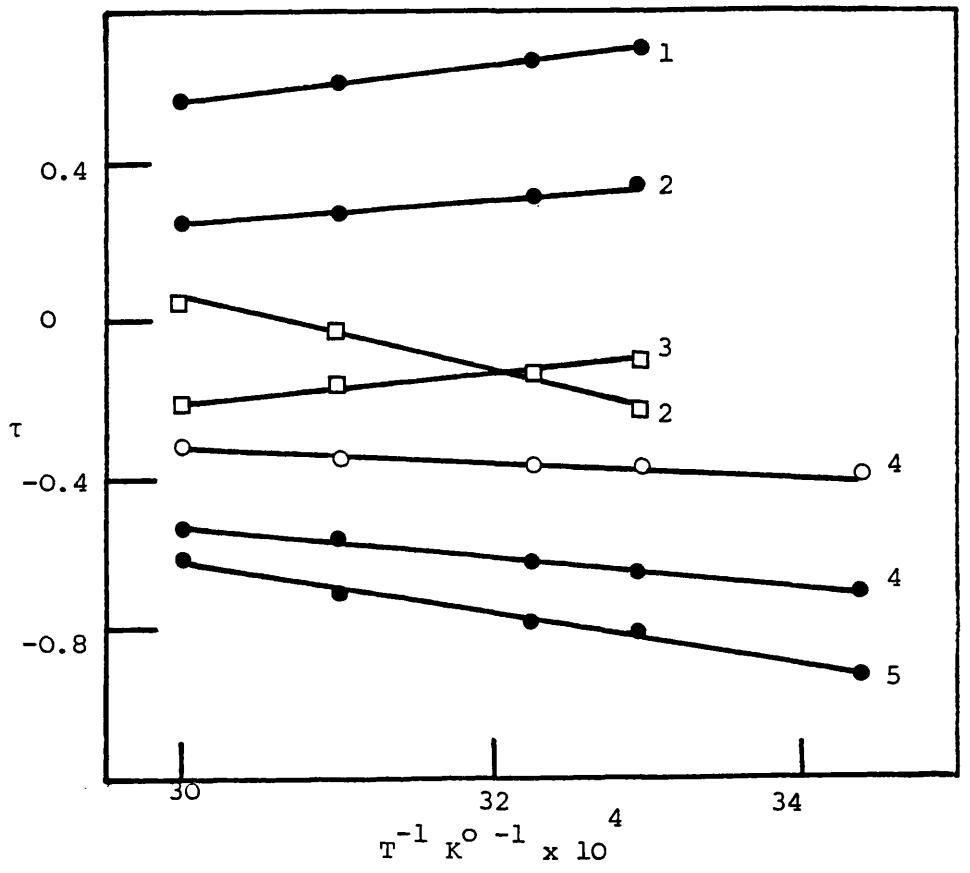
By application of Eq. 143 the extrathermodynamic terms, $\Delta(\Delta H^{o})$, $\Delta(\Delta S^{o})$ may be determined, since τ is independent of phase volume ratio (Section 1.2.2c). Despite the complex nature of HPLC systems it has been found that log κ (or τ) is linearly related to reciprocal absolute temperature (Fig. 4.23) over quite large temperature ranges, and that ΔH remains negative and constant even at temperatures close to $0^{o}C$ (228).

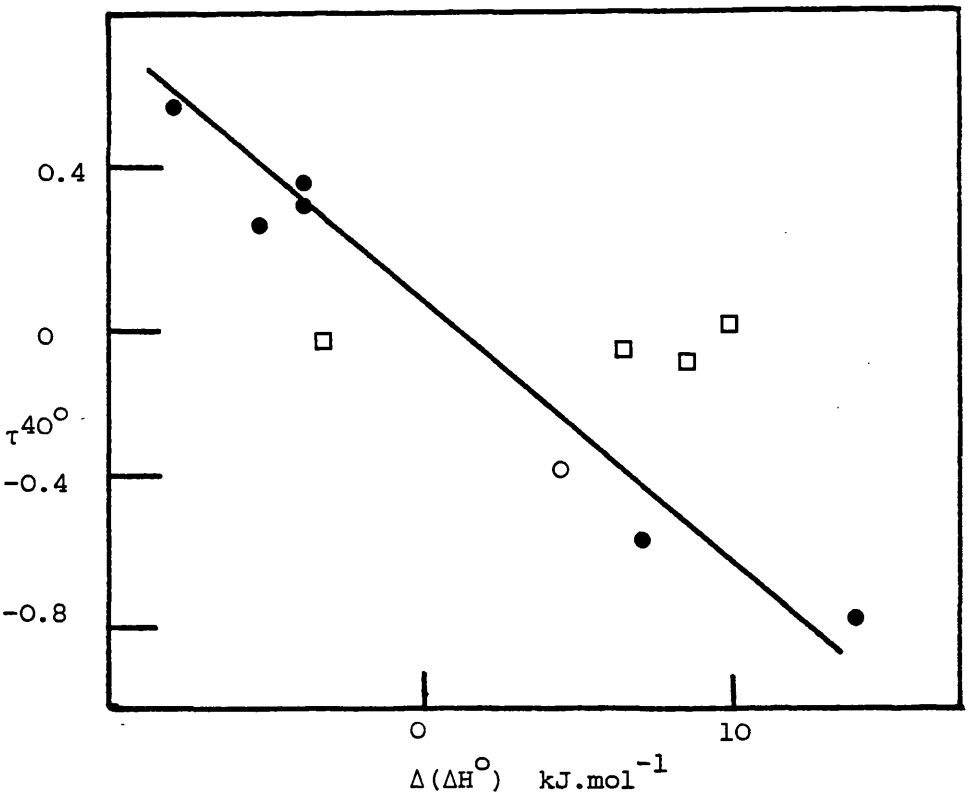
The capacity ratios of number of substituted benzoic acid analogues were determined using a Spherisorb ODS column with a mobile phase of C_{13}BDAC (5×10^{-4} mol.dm^{-3}) acetonitrile (20%) at pH 7.5 (K_2PO_4 2.5×10^{-2} mol.dm^{-3}) at different temperatures. Table 3.20 shows that the capacity ratios decrease with increasing temperature and the enthalpy changes obtained by analysis using Eq. 142 ranged from -15 to -45 kJ.mol^{-1}. Although the solute enthalpy terms were all negative, Table 4.12 and Fig. 4.24 show that the functional groups contribute both negatively and positiviely to this enthalpy term. Enthalpy values for other ion-pair HPLC systems can be obtained or calculated from the literature and range over approximately -16 to -35 kJ.mol^{-1} for amino acid solutes using alkylsulphate pairing-ions (165) to about -60 to -90 kJ.mol^{-1} for anionic dyes using small alkylammonium pairing-ions (227). These values and those presented here are generally higher than those reported for comparable non ion-pair systems (e.g. ref. 54).

The negative enthalpy values obtained in reversed phase HPLC systems are particularly intriguing since this is presumed to be effected by the same physicochemical process responsible for

Fig. 4.24. Van't Hoff plots, showing the relationship between τ and reciprocal absolute temperature. Table 3.21. Key as Fig. 4.4.

Fig. 4.25. Enthalpy-entropy compensation plot (τ^{T} - ΔΔH coordinates) (Eq. 149) for benzoic acid substituents derived from the harmonic mean temperature. Conditions as Table 3.20. Key as Fig. 4.4.





the hydrophobic process (regarded as being entropically driven and endothermic, that is positive ΔH and ΔS). It can be seen from Fig. 4.23 that the slopes of the Van't Hoff plots increase with increasing retention. Snyder (229)has recently described such systems as exhibiting 'regular' temperature behaviour such that the capacity ratios are controlled by the enthalpy of transfer. The dependence of capacity ratio on the enthalpy change may be given by differentiating Eq. 142. That is

$$\frac{d(\log k)}{d(1/T)} = \frac{-\Delta H^{o}}{2.3R} \qquad (144)$$

These 'regular' systems also exhibit enthalpy-entropy compensation behaviour since it is found that ΔH^{o} is linearly related to $T.\Delta S^{o}$, as expressed by:

$$\Delta H^{o} = \beta \Delta S^{o} + \Delta G^{o} \qquad (145)$$

where β is the compensation temperature. Leffler and Grunwald have shown (170) that to identify a single mechanism within a series of solutes, if ΔH and ΔS are approximated to be constant, $\delta\Delta H$ should be proportional to $\delta\Delta S$ (where δ denotes a change in the thermodynamic parameter by a medium effect, or as in this case a change in substituent). Thus it follows that linear enthalpy-entropy compensation may be demonstrated by the linear relationship

$$\Delta(\Delta H^{o}) = \beta \Delta(\Delta S^{o}) + \Delta(\Delta G^{o}) \qquad (146)$$

Others however (230, 231) have shown that this extrathermodynamic analysis leads to artifacts caused by statistical effects and not by true compensation. Accordingly the data presented here has been analysed by the method applied previously to non-ion-pair systems (228), such that in functional group terms (218) we may write:

$$\Delta(\Delta G^{\circ})_T = \Delta(\Delta H^{\circ})(1 - T./\beta) + T.\Delta(\Delta G^{\circ})/\beta \qquad (147)$$

and since:

$$\Delta(\Delta G^{\circ}) = - 2.3\ RT.\tau \qquad (148)$$

it follows that

$$\tau^T = \frac{-\Delta(\Delta H^{\circ})}{2.3\ R}\left[\frac{1}{T} - \frac{1}{\beta}\right] - \frac{\Delta(\Delta G^{\circ})}{2.3\ R\beta} \qquad (149)$$

where T is the harmonic mean temperature of the experimental values obtained, hence τ^T are group values measured at this temperature. The use of the harmonic mean temperature minimises statistical compensation. Figure 4.25 is the appropriate compensation plot between the functional groups values for τ, and $\Delta(\Delta H^{\circ})$ values calculated from the data given in Fig. 4.24 and listed in Table 4.12. With the exception of the *ortho* substituents linear enthalpy-entropy compensation is exhibited, as described by Eq. 150 to (where the *ortho* substituents are omitted), i.e.

$$\tau^T = -0.068\Delta(\Delta H^{\circ}) + 0.043 \qquad n=7,\ r=0.976 \qquad (150)$$

For the present systems studied this corresponds to a compensation temperature of about 770°K. This is in good agreement with Horváth *et al.* (228) who estimated that for reversed phase systems the narrowest estimated value of β is 554 to 755°K, and the results of Tomlinson *et al.* (232) who demonstrated that for neutral solutes in non ion-pair systems using aqueous methanol as an organic modifier in reversed phase HPLSC that the following was true, i.e.

$$\tau^T = -0.076\Delta(\Delta H^{\circ}) + 0.02 \qquad n=28,\ r=0.931 \qquad (151)$$

The deviation from linearity by the *ortho* substituents presumably reflects these steric and intramolecular effects discussed previously (Section 4.3.1) and suggests that for *ortho* substituted solutes entropy effects as well as enthalpy effects contribute to retention.

The good agreement between Eqs. 150 and 151 and the calculations of Horváth *et al.* (228) indicate that this relationship is a general one for reversed phase systems, at least when methanol-water or acetonitrile mobile phases are employed. To confirm this suggestion data has been obtained using a different stationary phase (SAS Hypersil), with benzoic acid and phthalic acid as solutes, and aqueous-acetonitrile mobile phases containing either C_{14}BDAC (10^{-3} mol. dm^{-1}) or a metal chelate (12-dien-Zn(II)) (10^{-3} mol.dm^{-1}) as pairing-ion. The results obtained (Tables 3.27 and 3.28) have been analysed according to Eq. 149 to obtain the appropriate extrathermodynamic functions, and these are presented as before by Eqs. 152 and 153, i.e.

C_{14}BDAC

$$\tau^T = -0.073\Delta(\Delta H^o)-0.06 \quad n=5,\ r=0.925 \qquad (152)$$

12-dien-Zn(II)

$$\tau^T = -0.064\Delta(\Delta H^o)-0.22 \quad n=5,\ r=0.899 \qquad (153)$$

The values used to generate Eqs. 152 and 153 are given in Tables 4.13 and 4.14. The slope coëfficients of approximately -0.07 indicate that the relationship is indeed a general one for all reversed phase systems studied, however the lower correlation coefficient obtained for the metal chelate system indicates that other factors, (probably in this case steric), tend to result in deviations. The metal chelate system showed significantly different selectivity differences from the ABDAC system particularly for the phthalic acids. Additionally the metal chelate system showed a greater dependence on temperature (i.e. higher enthalpy values) compared with the ABDAC systems.

A number of workers have shown that the decrease in retention

Table 4.12. Enthalpic and entropic group contributions and the τ values at the harmonic mean temperature. Conditions as Table 3.20.

Group	τ_{40}°	$\Delta(\Delta H^{\circ})$ ($kJ.mol^{-1}$)	$\Delta(\Delta S^{\circ})$ ($J.mol^{-1}K^{-1}$)
4-NH_2	-0.77	14.2	30.6
2-NH_2	-0.04	-3.22	-11.1
4-OH	-0.61	7.24	11.4
3-OH	-0.37	4.91	8.64
4-NO_2	0.31	-3.85	-6.29
2-NO_2	-0.17	7.65	21.2
4-CH_3	0.32	-5.51	-11.5
2-CH_3	-0.03	9.11	28.5
4-Cl	0.66	-8.16	-13.5
2-Cl	-0.06	6.50	19.6
CH=CH	0.39	-3.28	-2.91

Table 4.13. Enthalpic and entropic group contributions and τ^{40° values of some benzoic acids. Conditions as Table 3.23.

Group	τ^{40°	$\Delta(\Delta H^\circ)$	$\Delta(\Delta S^\circ)$
4-NH_2	-0.69	7.54	10.8
4-CH_3	0.29	-3.66	-6.02
2-COOH	0.07	0.80	3.98
3-COOH	0.06	-4.06	-11.8
4-COOH	-0.11	0.74	2.06

Table 4.14. Enthalpic and entropic group contributions and τ^{40° values of some benzoic acids. Conditions as Table 3.22.

Group	τ^{40°	$\Delta(\Delta H^\circ)$	$\Delta(\Delta S^\circ)$
4-NH_2	-0.57	3.48	0.27
4-CH_3	0.39	-3.30	- 3.16
2-COOH	0.87	-16.9	-37.4
3-COOH	0.29	-9.93	-26.1
4-COOH	-0.05	-5.23	-17.6

and selectivity due to increased temperature can be compensated for by a decrease in organic modifier concentration (86, 233). This can be demonstrated in this study by comparison of the slope parameters (ΔD) (Section 4.3.3) obtained for acetonitrile with the $\Delta(\Delta H^{o})$ in Table 4.12, i.e.

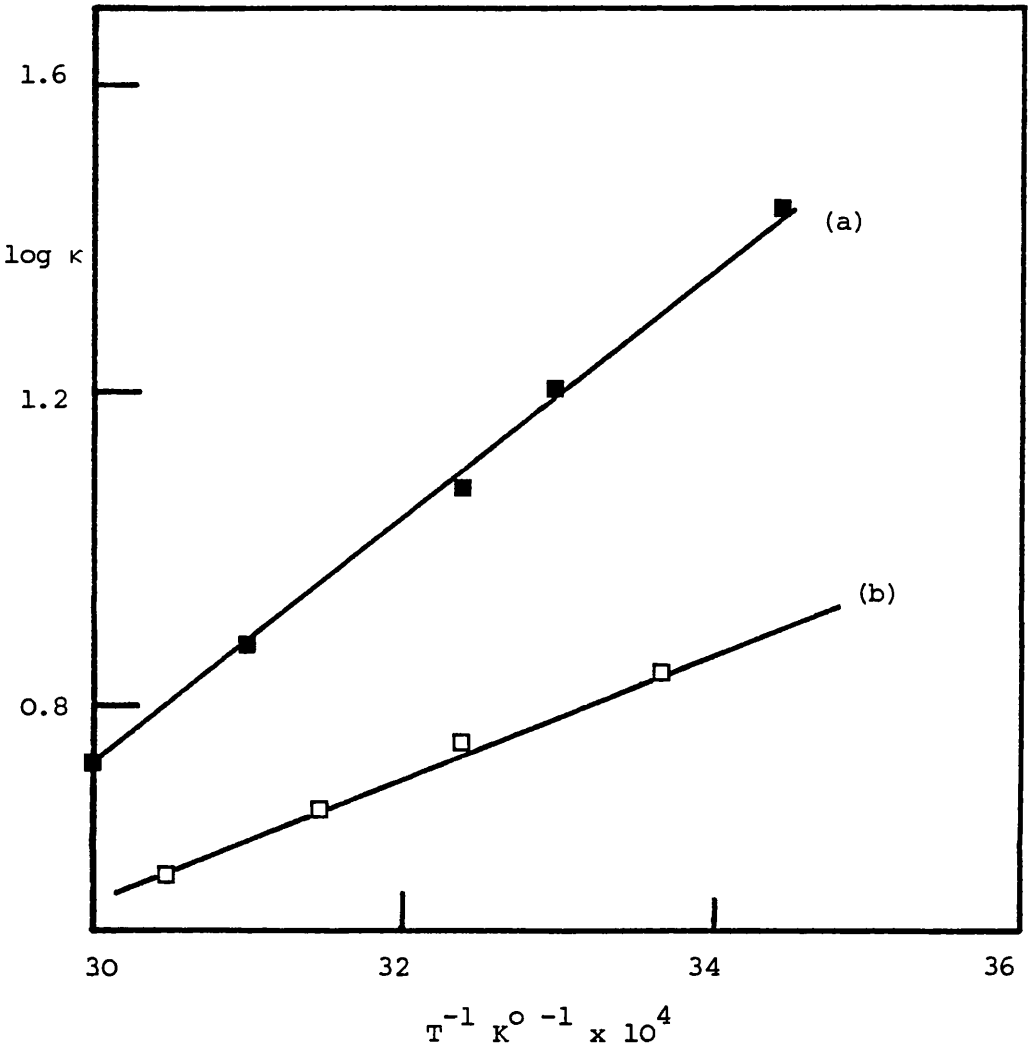
$$\Delta D = 0.28\ \Delta(\Delta H^{o}) + 0.04 \quad n=7,\ r=0.955 \qquad (154)$$

This concept can be appreciated further by reference to Fig. 4.26 which shows how the enthalpy change determines the retention of benzoic acid, an effect which has also been demonstrated in non-ion-pair systems (233). Consequently there seems to be little advantage in operating reversed phase ion-pair systems at elevated temperatures, however the increased column efficiency due to the improvement in mass transfer will have a beneficial effect on resolution. Table 4.15 shows the dramatic decrease in plate height with increasing temperature, observed in this study.

Table 4.15. The effect of temperature on the plate height (H) of 3-hydroxybenzoic acid. Conditions as Table 3.20.

Temperature (^{o}C)	H (mm)
18.8	0.097
31.0	0.069
38.2	0.048
50.6	0.038
60.5	0.032

Fig. 4.26. Van't Hoff plots for benzoic acid showing log κ *versus* reciprocal absolute temperature, T^{-1} and the dependence of retention of standard enthalpy change. Conditions: (a) as Table 3.20 and (b) as Table 3.22.



4.3.6 Stationary phase material

The retention behaviour and selectivities of five alkyl silica stationary phase materials were compared under standard mobile phase conditions. The surface characteristics of the stationary phases chosen were first determined and the results are presented in Table 2.10. It can be seen that the surface areas of the materials vary from about 200 $m^2.g^{-1}$ for the spherical materials (Hypersil and Spherisorb) to 400 $m^2.g^{-1}$ for the irregular materials (Partisil). A higher pressure was required to produce the same flow rate for the irregular particles compared with the spherical materials, this is reflected in the values of the effective diameters (Table 2.8) obtained during column testing.

With respect to reversed phase chromatographic behaviour the important surface characteristics are the carbon chain length of the bonded alkyl groups, the carbon loading and the presence of residual silanol groups which may contribute to secondary retention mechanisms. Four octadecylsilicas and a hexylsilica have been compared with respect to retention and selectivity. Table 2.10 shows the retention behaviour of nitrobenzene eluted with a mobile phase of dry n-hexane. It can be seen that significant silanol groups are present for the two Partisil materials and Spherisorb S5 ODS whereas ODS Hypersil and Spherisorb Hexyl were fully capped. These results are in agreement with the manufacturers specifications. The carbon loadings of the stationary phase materials were determined by elemental analysis and ranged from 3.9% w/w for Partisil 10 ODS to 15% w/w for Partisil 10 ODS 2. The very high carbon loading of the latter materials is indicative of C-C cross linkages between the alkyl chains and this

material may be considered to be partially polymeric in nature (28). These cross-linkages and higher surface coverage of Partisil 10 ODS 2 had little effect on the accessability of the residual silanolol groups, since the retention of nitrobenzene was greater in this phase compared with Spherisorb S5 ODS, which is partially capped but has a lower surface coverage of alkyl groups.

Table 3.26 gives the capacity ratios of some substituted benzoic acids, phenylacetic acid and cinnamic acid using these five stationary phase materials with a standard mobile phase of 50% v/v methanol, and 5×10^{-4} mol.dm^{-3} C_{14}BDAC at pH 7.5 (2.5×10^{-2} mol.dm^{-3} K_2HPO_4). The retentive powers of the octadecyl stationary phases are ranked Partisil 10 ODS 2 > ODS Hypersil > Spherisorb S5 ODS > Partisil 10 ODS and are directly related to the carbon loading of the stationary phases. For the hexyl material the retention of the benzoic acid solute fitted a ranking based on carbon loading, however with respect to some of the solutes this order did not always apply indicating that the shorter alkyl chain produced different selectivities.

To determine the selectivities of the phases the retention behaviour of the solutes was compared with ODS Hypersil as the reference stationary phase. The behaviours can be conveniently analysed in terms of a simple correlation expression, i.e.

$$\log k_x = J.\log k_{\text{ODS Hypersil}} + K \qquad (155).$$

where x refers to the test stationary phase.

A value of one for the slope coefficient, J, means that there is no contribution of the stationary phase to selectivity compared

to Hypersil. Table 4.16 gives the appropriate regression coefficients of Eq. 155 for the various phases. It can be seen that J varies significantly from unity (at the 99% level of significance), and for the octadecyl stationary phases J increases with carbon loading.

Table 4.16. Regression coefficients of Eq. 155 comparing the selectivities of four stationary phase materials with ODS Hypersil. Conditions as Table 3.26.

Stationary phase	J	K	r
Spherisorb S5 ODS	0.876	-0.101	0.985
Spherisorb hexyl	0.841	-0.073	0.997
Partisil 10 ODS	0.808	-0.320	0.964
Partisil 10 ODS 2	1.178	0.259	0.994

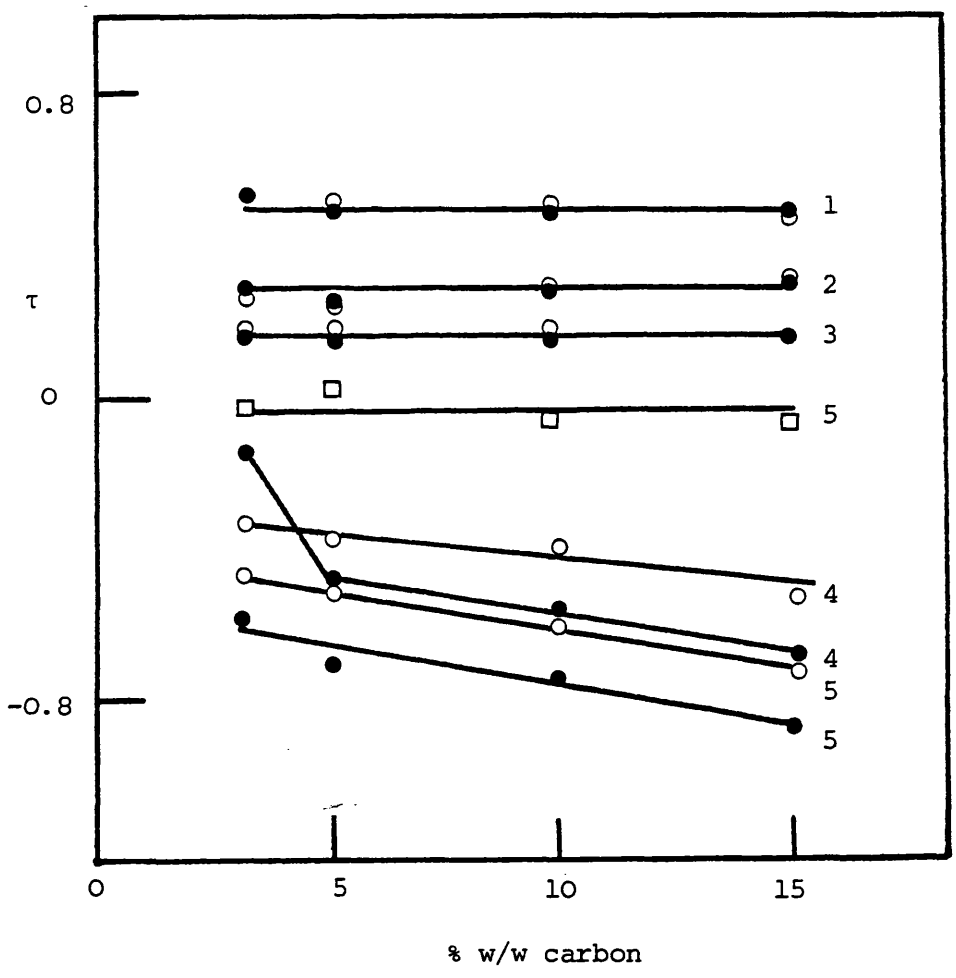
The value of J for the hexyl is lower than would be expected on the basis of carbon loading, suggesting that smaller alkyl chain length materials are less selective than the longer alkyl chain materials. The high correlation coefficients are indicative of a common retention mechanism.

The effect of the stationary phase on selectivity is further demonstrated by applying the general relationship relating τ to π (Eq. 139) to functional group values obtained using the five stationary phases (Table 3.27). Table 4.17 gives the regression coefficients according to Eq. 139, and it can be seen that the slope coefficient increases with carbon loading with the exception

Table 4.17. Regression coefficients for Eq. 139 relating τ to π under standard mobile phase conditions, using five different alkyl silicas. Conditions as Table 3.26.

Stationary phase	H	I	r
ODS Hypersil	0.60	0.04	0.976
Spherisorb S5 ODS	0.52	0.02	0.972
Partisil 10 ODS 2	0.69	-0.01	0.974
Partisil 10 ODS	0.50	0.10	0.979
Spherisorb Hexyl	0.46	0.04	0.972

Fig. 4.27. Effect of octadecyl stationary phase carbon loading on benzoic acid τ values. Conditions as Table 3.26. Key as Fig. 4.4.



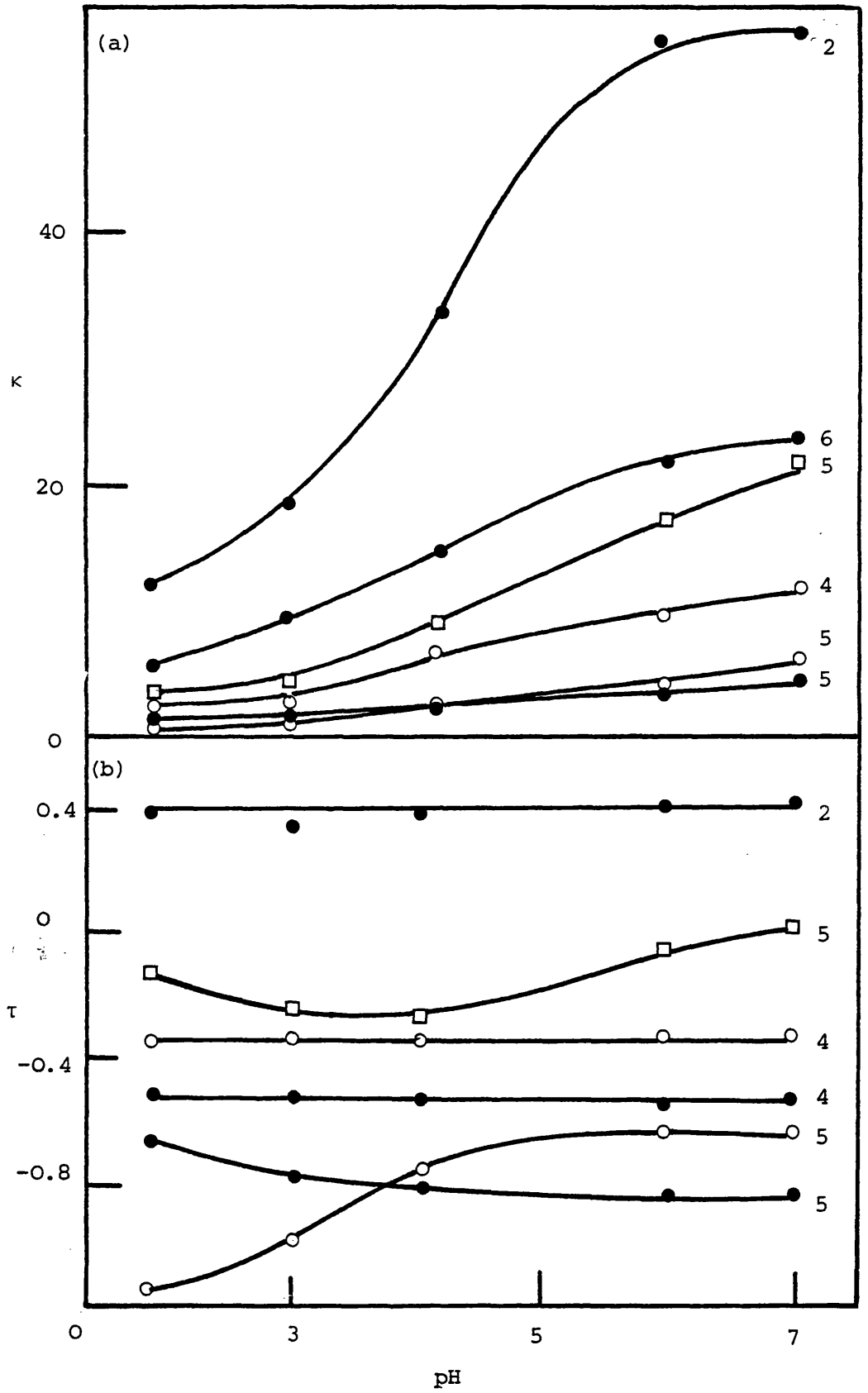
of the hexyl material, which has a lower value than would be expected. The high correlation coefficients again indicate that solvophobic forces dominate the retention process in all cases. The significant positive intercept for the Partisil 10 ODS data can be attributed to the effect of residual silanolol groups on the τ value for the 4-OH function. It can be seen that with the low carbon loading, uncapped material the elution order of 3- and 4-hydroxybenzoic acids is reversed presumably due to the interaction of the unhindered 4-OH group with the silica surface.

Inspection of τ values (Table 3.27) shows that stationary phase carbon loading only affects the polar groups, and that for solvophobic groups (positive τ) values are independent of carbon loading. This effect is demonstrated graphically by Fig. 4.27. Hennion *et al*. (77) have demonstrated that selectivity increases with stationary phase carbon loading up to about 15%, above which it remains fairly constant. These results of Hennion *et al*. and those presented here, give us confidence in suggesting that τ values will be interconvertable for the more modern packing materials having a maximum surface coverage of carbon with no C-C cross linkages and residual silanolol groups.

4.3.7 pH

It has been shown here and elsewhere (Section 1.5.3) that although retention of ionised solutes is enhanced by the addition of oppositely charged pairing-ions, solute retention and selectivity are still controlled by solvophobic forces. The charge on the solute only influences retention and selectivity due to the stoichiometry of interaction. If solvophobic effects dominate

Fig. 4.28 Effect of mobile phase pH on benzoic acid capacity ratios, κ, (a) and τ values (b). Conditions as Table 3.28. Key as Fig. 4.4.



in ion-pair systems then the functional group values obtained should be the same as those obtained when the solute is unionised and retained by a process which does not involve ion-pairing.

To confirm this hypothesis, the capacity ratios of several substituted benzoic acids have been measured at a constant pairing-ion concentration at different pHs. To minimise the effect of changes in ionic strength a high concentration of potassium nitrate was employed in the mobile phase rather than potassium phosphate, (the ionised forms of which change in proportion with changing pH (224)). Fig. 4.28a shows that while retention is dependent on mobile phase pH, (with retention being higher when retention is due to ion-pair formation), functional group values are independent of pH, so confirming the hypothesis that the same factors control selectivity in both ion-pair and non ion-pair reversed phase systems. (The change in the τ value for the amino groups is due to protonation and not a change in retention mechanism). The close proximity of the pKa values of the solutes chosen minimises any dependence of τ on pH that might be seen when the pH of the mobile phase is close to the pKa of the solute.

The behaviour of the aminobenzoic acids has prompted us to investigate the effect of pH on the retention behaviour of a number of amphoteric solutes in the presence of either cationic or anionic surfactants. Accordingly Fig. 4.29a shows the effect of pH on the retention of 4-aminobenzoic acid both in the absence of a pairing-ion and the presence of either SDDS or C_{11}BDAC (both at 5×10^{-4} $mol.dm^{-3}$). Because of the similar pKa and pKb values of amino-benzoic acid, the κ *versus* pH relationship in the absence of a

pairing-ion shows a sharp maximum around the iso-electric region of pH 3.5 to 4.0. Similar effects were observed with the other aminobenzoic acids. Since these are amphoteric solutes they could ion-pair with either the anionic or cationic surfactant, depending on the pH. At low pH the protonated ampholyte can ion-pair with the added SDDS,and also as the pH is raised from 2 to 6 the ampholyte species dominates as the anion and can be ion-paired with the added cationic species (C_{11}BDAC). Above pH 6 a fall in solute retention was observed, which can be postulated as being due to the presence of the phospahte buffer components, since this effect was not observed when nitrate was the added inorganic counter-anion. Around pH 6 there is a change in profile corresponding to the change from dihydrogen to monohydrogen phosphate species. It is likely that monohydrogen phosphate ions will associate far more readily with the C_{11}BDAC than the dihydrogen species, (due to its increased charge number), so resulting in a loss in the thermodynamic activity of the pairing-ion and a reduction in retention.

Due to pH limitations in the use of the reversed phase materials (pH 2 - 7.5), the maxima exhibited by aminobenzoic acids were not observed for the aminopropionic acids since their pKa values are much higher (Table 4.18). As a result the aminopropionic acids only pair with the anionic surfactant (SDDS) at pHs below 4.5, as predicted from knowledge of their pKb values as demonstrated by Fig. 4.29b. Histidine has two pKbs (7.9 and 11.4) and as a result is expected to form 2:1 complexes at pH 2 and only 1:1 complexes with SDDS at higher pHs. This is suggested to be the case from the pH *versus* κ profile for histidine (FIg. 4.30), which

shows two distinct breaks in the curve at pHs equal to a value of 14 - pKb. It has been found also that the phosphate ions influence the retention of the aminopropionic acids. In contrast with the aminobenzoic acids phosphate acts as a pairing-ion at low pH rather than as a competing ion at high pH, which can be seen by the decrease in retention for the aminoproprionic acids in the absence of any pairing-ion between pH 2 and 4. At low pH in the presence of C_{11}BDAC the retention is reduced, in this case it is the C_{11}BDAC which acts as the competing ion reducing the thermodynamic activity of the phosphate compared to the converse effect occurring at high pH.

These pH effects in ion-pair systems may be predicted quantitatively by modification of Eq. 53 to take into account the effect of ionisation and retention of the unionised species (234). Eq. 53 now becomes:

$$\kappa = (\kappa^{o}+K_1^{o}K_a/[H^+] + K_2K_4[X])(1+Ka/[H^+]+K_2[X])^{-1} \quad (156)$$

where Ka is the acid dissociation constant, $[H^+]$ is the hydrogen ion concentration, K_1^{o} represents the solute-stationary phase binding constant of the uncharged species, and $[X]$ is the pairing-ion concentration. The data obtained here has been solved in terms of Eq. 156 by using literature values of K_a to yield the ion-pair formation constants (K_2) and the ion-pair distribution constants (K_4). As shown previously (Section 4.3.1) the ion-pair distribution constants can be related to measures of solute hydrophobicity (i.e. Σf, ref. 235) as shown by Fig. 4.31, which adds further weight to the hypothesis that solvophobic forces dominate in these systems, and that retention is due to ion-pair distribution.

Fig. 4.29. The effect of mobile phase pH on the capacity ratios, κ, of (a) 4-ABA and (b) 5-HTP, in the presence of either SDDS (5×10^{-4} mol.dm^{-3}) or C_{11}BDAC (5×10^{-4} mol.dm^{-3}). Tables 3.30 to 3.32.

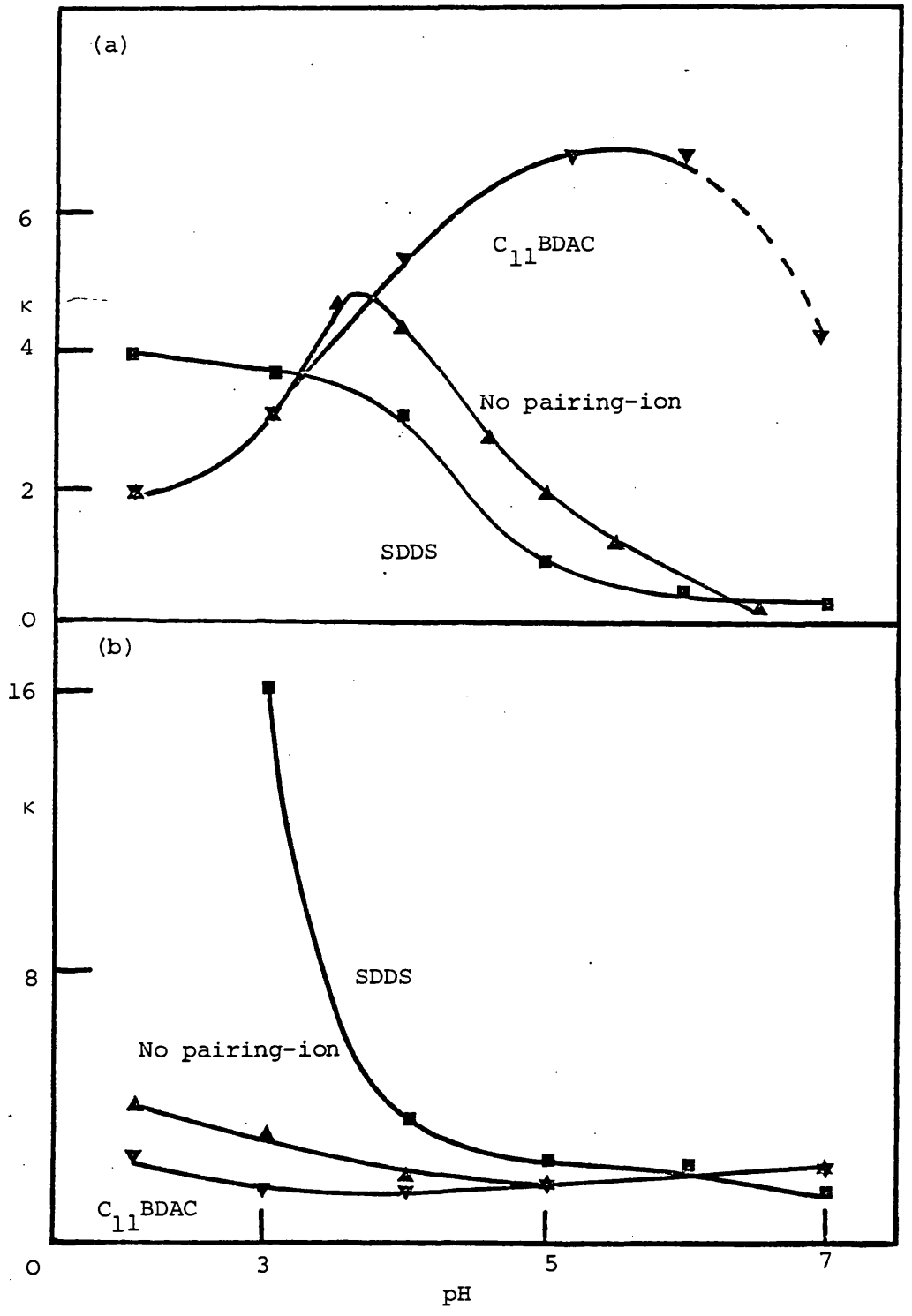


Table 4.18. Literature and chromatographically determined physicochemical constants.

Solute	Dissociation constants			Σf (c)	K^o	K_2	K_4
	pKa (a)	pKb (a)	pKb (b)		($mol.^{-1} dm^3$)		
Ampholytes							
His	9.18	7.90,11.42	8,1,10.5	-0.23	-	-	-
DOPA	9.24	11.4	11.5	1.52	0.67	134	74.1
Phe	9.24	11.4	11.5	2.24	1.40	551	123
CPA	9.24	11.4	11.5	3.23	7.40	369	765
5-HTP	9.39	11.6	11.5	1.95	1.30	1130	33.9
Trp	9.39	11.6	11.5	2.31	5.3	344	396
2-ABA	2.09	4.8	4.6	1.65	0.90	2303	18.6
3-ABA	3.08	4.8	4.6	1.65	0.20	1983	12.6
4-ABA	2.50	4.8	4.7	1.65	0.30	3335	5.9

Monopoles		pKa (b)
2-NBA	2.17	3.5
3-HBA	3.00	3.5
4-HBA	4.57	4.5
3-MBA	4.23	4.5
4-MBA	4.37	4.5
BA	4.40	4.5

a. Literature values (228)

b. This study

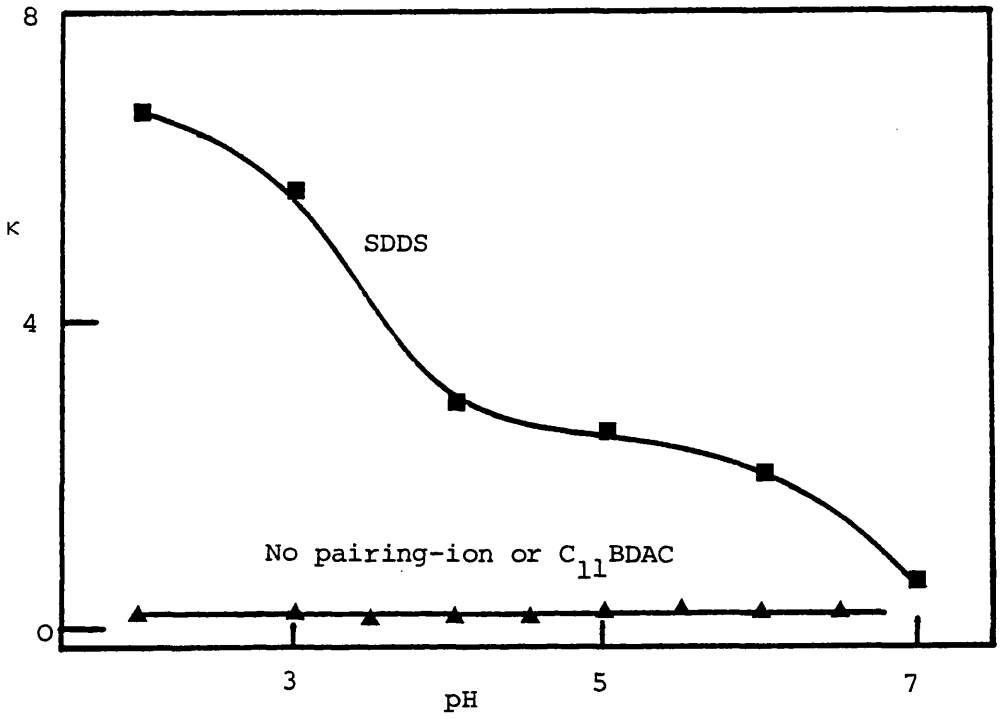
c. Relative hydrophobicities of the uncharged portions of the molecules computed from values given by Rekker (235).

Operationally selectivity in reversed phase ion-pair systems may be controlled by changing the pH if the solutes have different pKas, or different charge numbers in which case the different stoichiometry of ion-pairing may be utilised. For example consider either the retention order of 3- and 4- aminobenzoic acids which is reversed both in the presence of either cationic or anionic surfactants (Fig. 4.32), or the reversal in the elution order of DOPA and histidine at pH 2 to 5 in the presence of SDDS.

From the pH profiles of the amino acids it is possible to determine the pK values of the aminoproprionic acids and these are given in Table 4.18; agreement between literature (228) and experimental values is good which both indicates that the assumed mechanism of retention is an appropriate one and that at the low organic modifier concentrations used no pH correction factor is needed to account for possible change in the hydrogen ion activity (236).

Fig. 4.30. Effect of mobile phase pH on the capacity ratio of Histidine in the presence of SDDS (5×10^{-4} mol.dm^{-3} or C_{11}BDAC (5×10^{-4} mol.dm^{-3}). Tables 3.30 to 3.32.

Fig. 4.31. Relationship between the chromatographic ion-pair distribution constant, K_4 (Eq. 184) of some amino acid and Σf. Table 4.16.



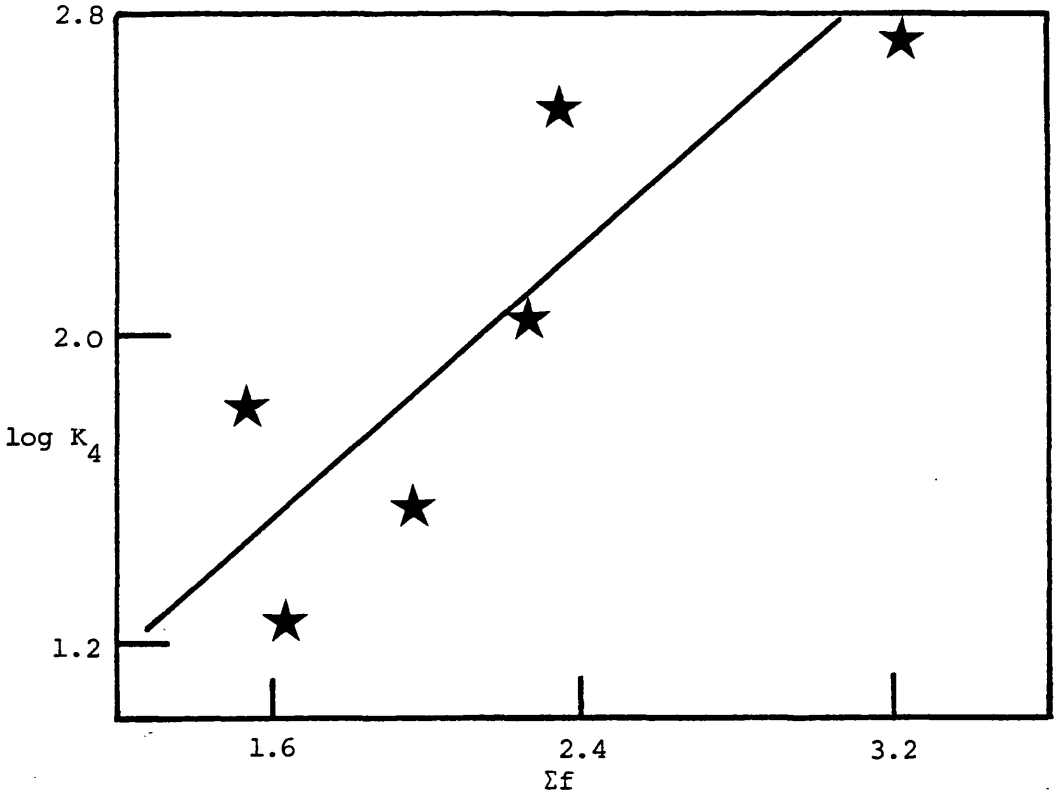
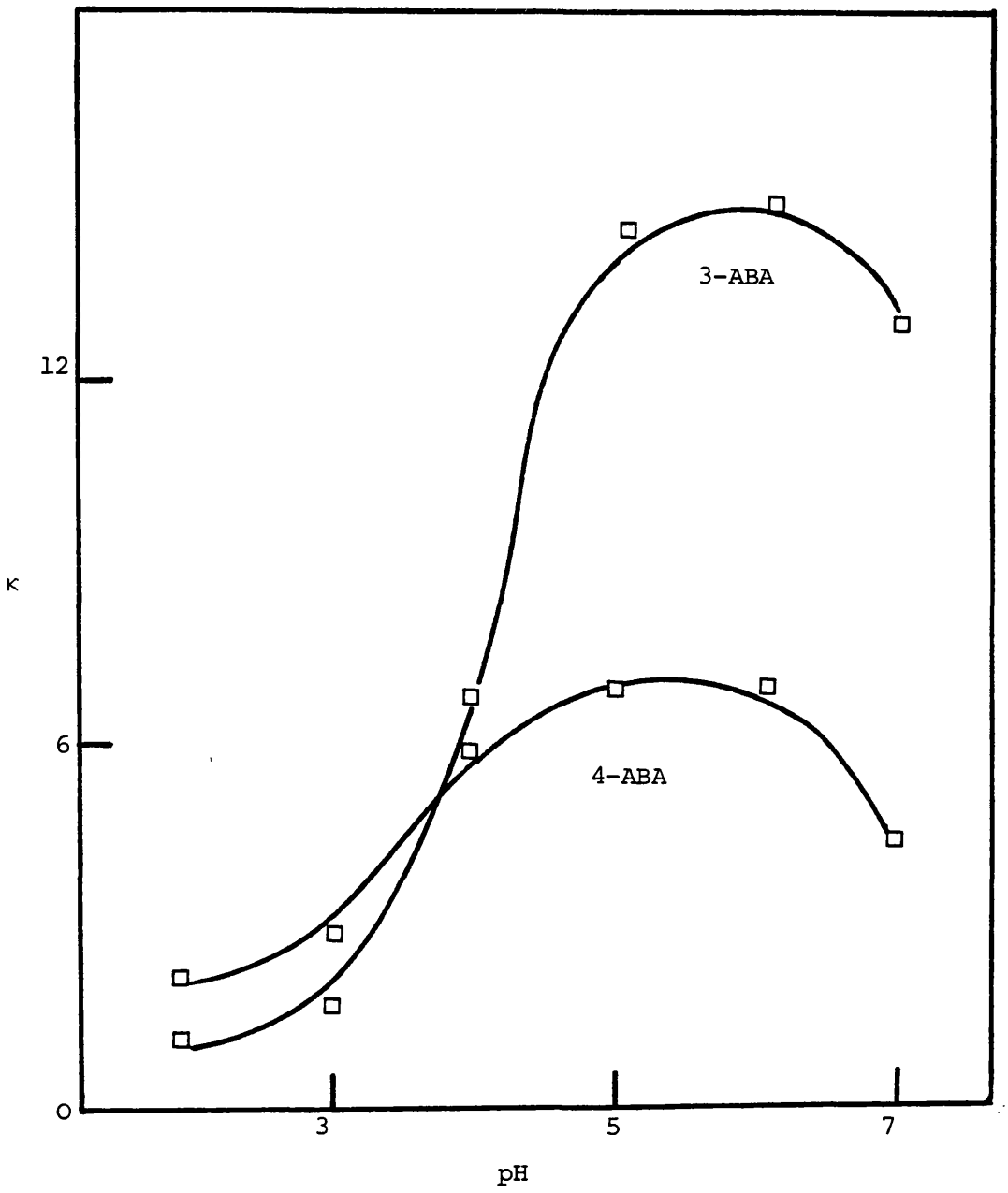


Fig. 4.32. κ *versus* mobile phase pH for 3-ABA and 4-ABA showing reversal of retention order at higher pH in the presence of a cationic pairing-ion. Conditions as Table 3.32.



4.4 Applications

The results presented here and elsewhere have shown that retention in reversed phase ion-pair HPLSC is controlled primarily by Solvophobic forces, and that desired retention and selectivity may be achieved by a rational choice of the organic modifier type and concentration. The possibility of controlling the concentrations and hyrophobicities of the pairing-ions provides added flexibility compared with non ion-pair systems. Although selectivity is apparently independent of pairing-ion hydrophobicity and concentration, the addition of a pairing-ion increases the resolution of ionised solutes due to the increase in retention of the solutes. A notable exception to this latter rule is in the separation of solutes of different charges when selectivity is influenced by the stoichiometry of ion-pairing and in this case selectivity may be further controlled by changing the alkyl chain length of the pairing-ion and/or the pH of the mobile phase. Practical analytical problems often require the separation of neutral and ionised solutes in which case the addition of pairing-ion has a dramatic effect on resolution since selective increases in the retention of the ionised solutes are possible. In the following sections the applicability and extreme flexibility of approach found using ion-pair systems with surfactant pairing-ions is described.

4.4.1 Acidic metabolites of tryptophan, phenylalanine and histidine.

In sections 4.3.1, 4.3.2 and 4.3.3 it was shown that required retentions may be obtained by controlling the concentration and type of the pairing-ion and the concentration of the organic modifier. The interrelationship between these two parameters has been

examined by investigating the retention behaviour of eight acidic metabolites of three amino acids in mobile phases containing 0 to 5×10^{-4} mol. dm^{-3} C_{14}BDAC and 15 to 25% v/v acetonitrile in an aqueous mobile phase at pH 7.5.

Figure 4.33 shows typical results (for HMA and HPPA) for the relationship between capacity ratio and C_{14}BDAC concentration at three different acetonitrile concentrations. As before (Section 4.3.1) there is an initial sigmoidal effect, followed by a hyperbolic relationship between capacity ratio and paring-ion concentration. Above a pairing-ion concentration of 2×10^{-4} mol.dm^{-3} C_{14}BDAC, decreasing the acetonitrile concentration increased the retention of all the solutes, although below this concentration retention was either constant or reduced. Different combinations of pairing-ion and acetonitrile concentrations resulted in changes in selectivity and retention order. Figure 4.34 shows that moving from a mobile phase containing 5×10^{-4} mol.dm^{-3} C_{14}BDAC and 20% v/v acetonitrile, to one containing 2×10^{-4} mol.dm^{-3} C_{14}BDAC and 15% v/v acetonitrile results in similar overall analysis times but causes a reversal in the retention orders of 3-hydroxyanthranillic acid and 3,4-dihydroxymandelic acid and of homogentisic acid and 4-hydroxyphenypyruvic acid, due to changes in selectivity.

The selectivity of C_{14}BDAC has been compared with the metal chelate pairing-ion 12-dien-Zn(II) (207) using an aqueous mobile phase containing 20-30% v/v acetonitrile at pH 7.1 (0.1 mol.dm^{-3} (NH_4Ac). Figure 4.35 shows that the retention of the acids decreases with increasing acetonitrile concentration however there were noticeable differences in selectivity compared with

Fig. 4.33. The relationship between the capacity ratios of (a) HMA and (b) HPPA and pairing-ion concentration (C_{14}BDAC) at three acetonitrile concentrations. Conditions as Tables 3.33 to 3.35. The numbers refer to the percentage (by volume) of acetonitrile.

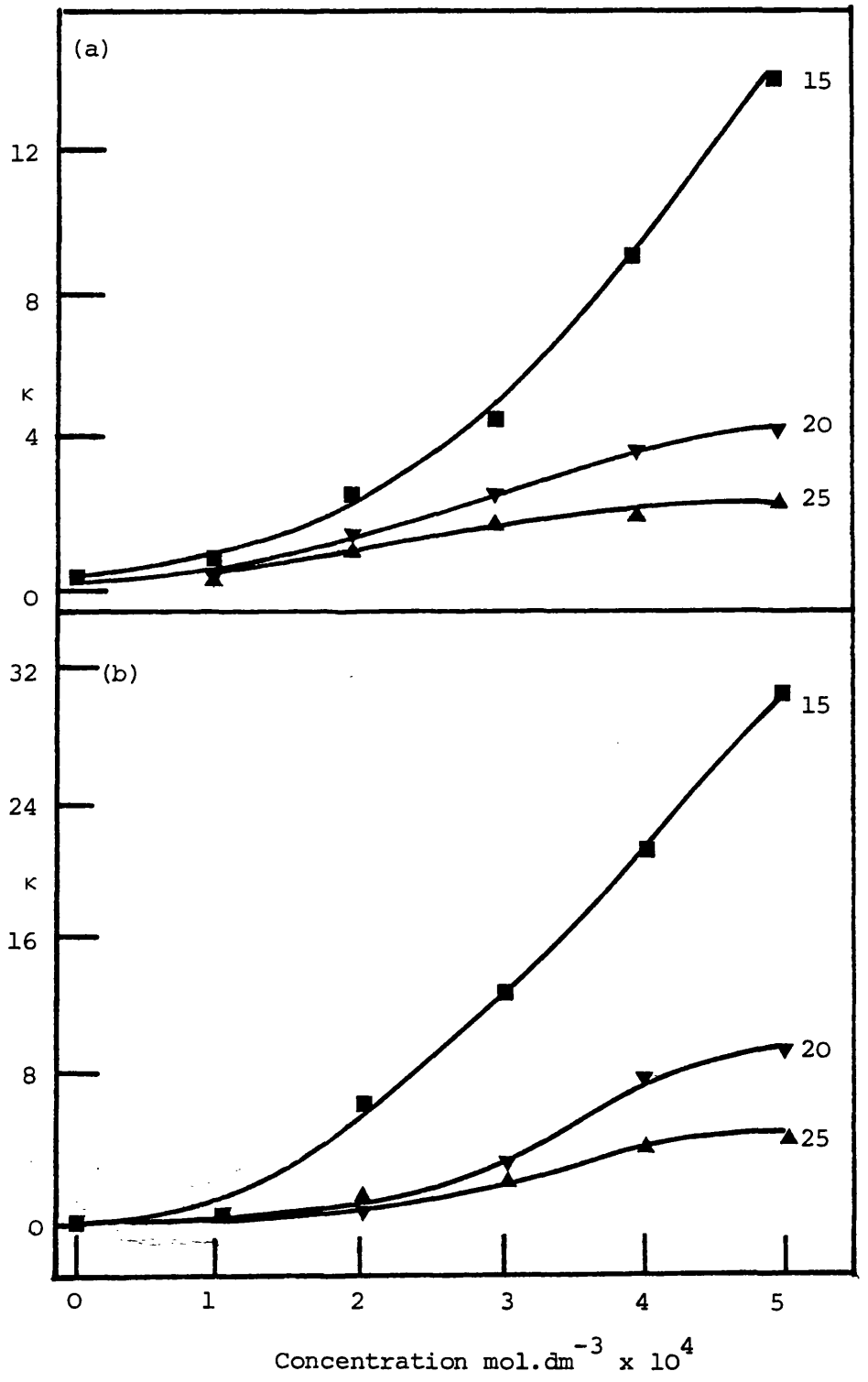


Fig. 4.34a. Separation of some acidic metabolites of tryptophan, histidine and phenylalanine. Stationary phase: ODS Hypersil. Mobile phase: 20% v/v acetonitrile, 5 x 10^{-4} C_{14}BDAC, pH 7.5 Temperature: 30°C. Key: 1, IAA; 2, UA; 3,HMMA; 4,HMA; 5,HAA; 6, 5-HIAA; 7, HPPA; 8, HG.

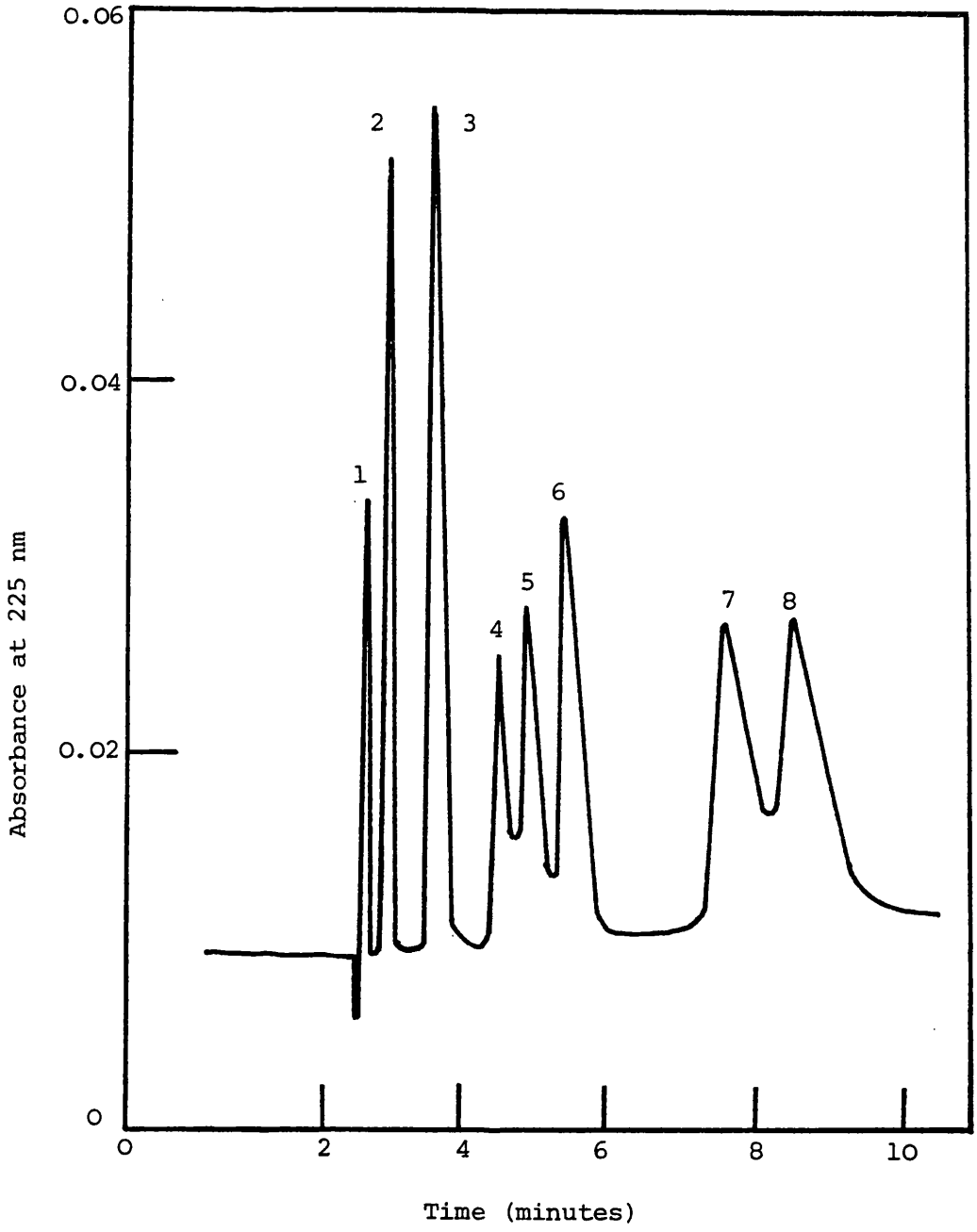


Fig. 4.34b. Separation of some acidic metabolites of tryptophan, histidine and phenylalanine. Stationary phase: ODS Hypersil. Mobile phase: 15% v/v acetonitrile, 2×10^{-4} mol.dm^{-3} C_{14}BDAC, pH 7.5. Temperature: 30°C. Key as Fig. 4.34a.

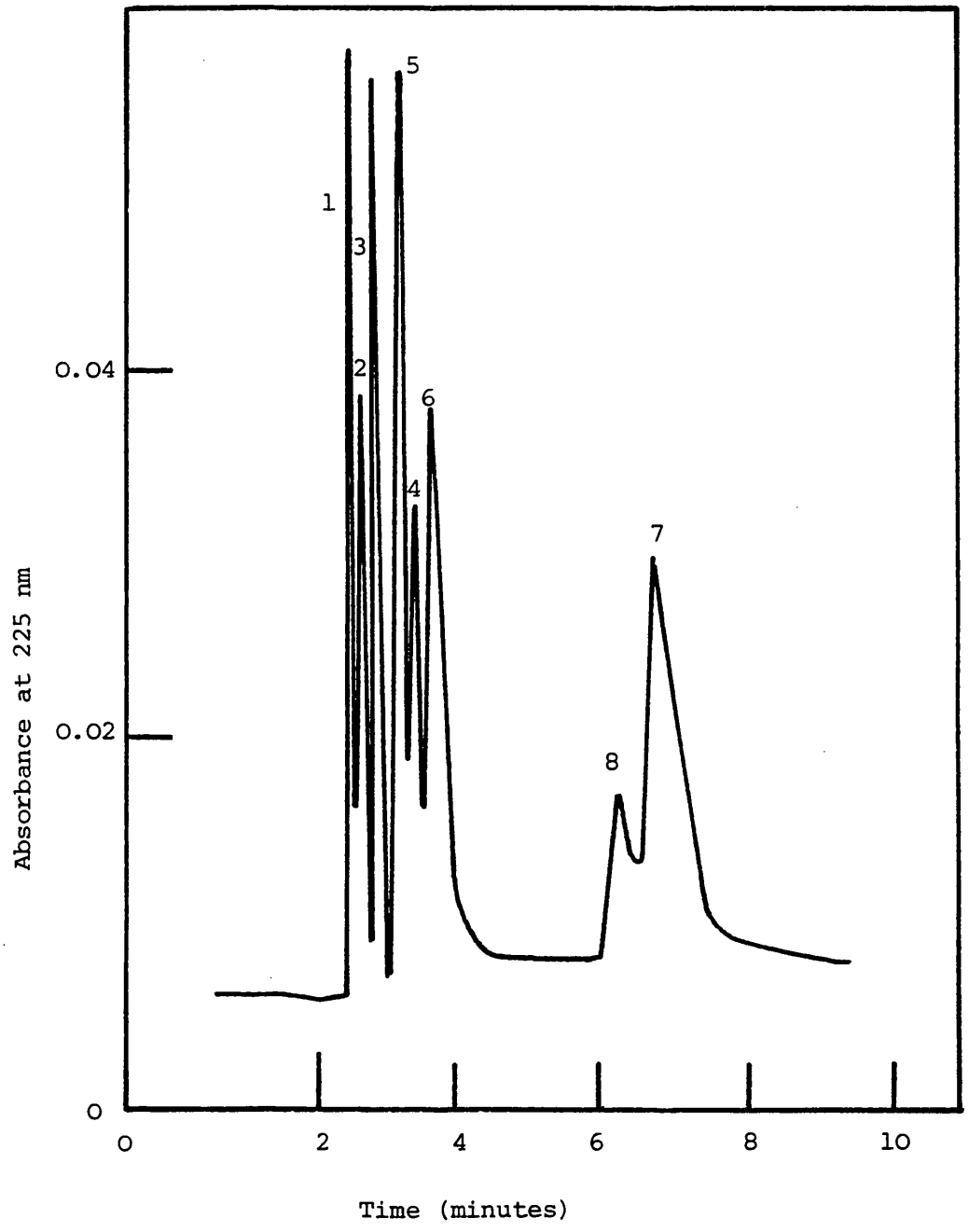


Fig.4.35. The effect of acetonitrile concentration on the capacity ratios, κ, of the active metabolites of tryptophan, histidine and phenylalanine. Conditions as Table 3.36. Key as Fig. 4.34a.

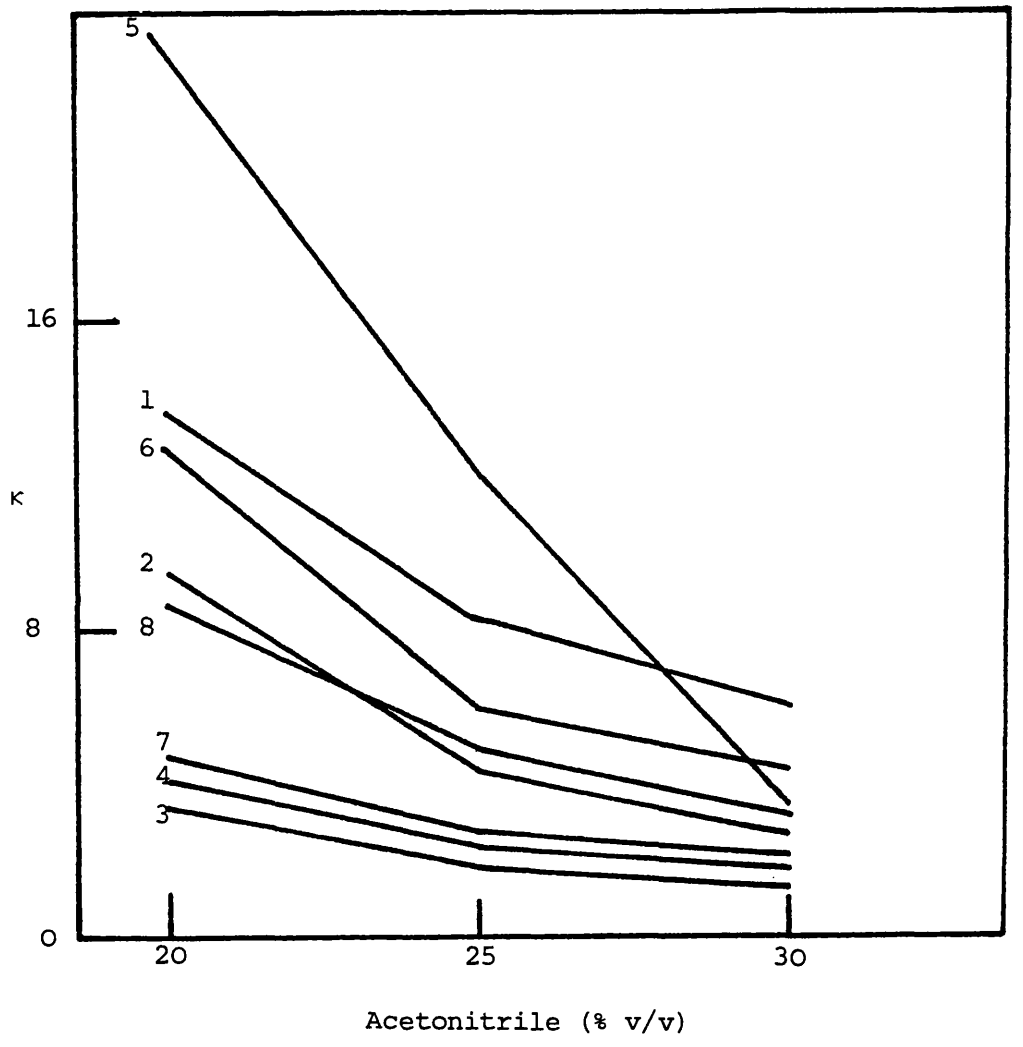
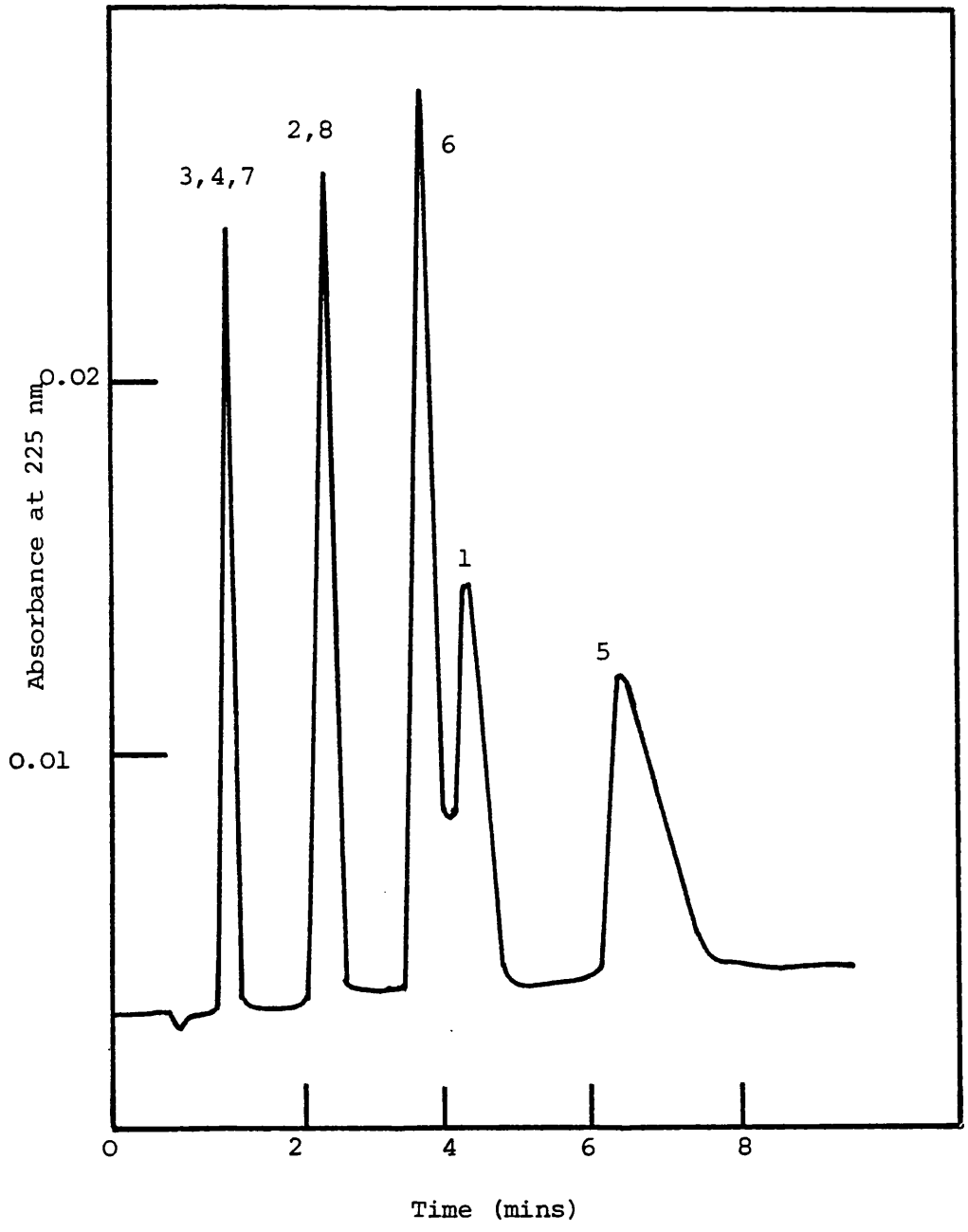


Fig. 4.36. Separation of some acidic metabolites of tryptophan, histidine and phenylalanine. Stationary phase: ODS Hypersil, Mobile phase: 25% v/v acetonitrile, 0.13 mol.dm^{-3} ammonium acetate, 10^{-3} mol.dm^{-3} 12-dien-Zn(II), pH 7.1. Temperature: 30°C. Key as Fig. 4.34a.



C_{14}BDAC, as given by Fig. 4.36 which gives the separation of the acids in a mobile phase containing 12-dien-Zn(II) 10^{-3} mol.dm^{-3}) and 20% v/v acetonitrile. The most noticeable change in selectivity is observed for the imidazole acids for which retention times were increased considerably and their retention order reversed. Complete separation of the acids was not possible using the 12-dien-Zn(II) pairing-ion, partly due to the reduced selectivity but mainly due to the reduced column efficiencies achieved. For a 95 mm column the 12-dien-Zn(II) system gave 1000 theoretical plates compared with 3000 for the C_{14}BDAC system.

4.4.2 Tryptophan in biological fluids

Tryptophan blood levels have been considered to be indicative of mood changes in man and there have been several reports of the benefits of tryptophan in the treatment of depression (237). Tryptophan is found in significant amounts in saliva and it has been suggested (238) that there is an equilibrium between the free tryptophan in plasma and in saliva.

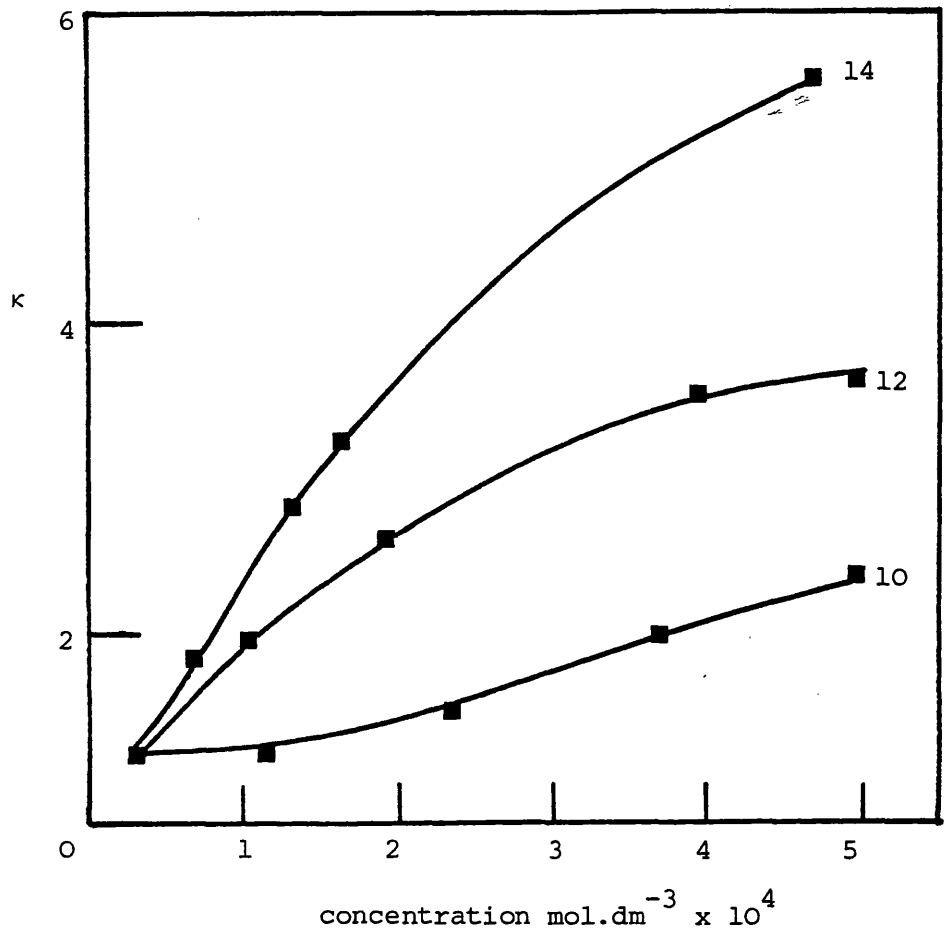
A variety of methods have been employed for the determination of tryptophan in biological fluids, including thin-layer chromatography (239), amino acid analysis (15, 240, 241), gas chromatography (242, 243), UV spectrometry (244) and fluorimetry (245). These methods have proved to be insensitive, tedious and/or fairly non-selective. Recently reversed phase HPLC (206, 246) and ion-pair HPLC (247) methods have been presented for the determination of tryptophan in biological fluids and proved to be highly selective. An advantage of reversed phase HPLC is that the retention of solutes is determined primarily by their solvo-

phobic character, thus polar endogenous materials and drug metabolites elute rapidly in a narrow band. By the application of ion-pairing techniques it is possible to enhance selectively the retention of ionised solutes which would normally elute with this band of endogenous materials.

Chromatographic conditions have been optimised by investigating the effect of alkylsulphate pairing-ion concentration and chain length on the retention of ionised tryptophan using a Spherisorb S5 ODS stationary phase and a mobile phase of methanol: water (1:1) at pH 2.25. A sigmoidal relationship between capacity ratio and pairing-ion concentration was obtained (Fig. 4.37). The logarithm of the capacity ratio can be related linearly to the number of carbon atoms in the alkyl chain of the pairing-ion. The methylene group contribution of the pairing-ion to retention was independent of concentration above 2×10^{-4} $mol.dm^{-3}$ alkylsulphate, the slopes of log k *versus* n above this concentration (Eq. 114) were constant (0.11 ± 0.03), and were consistent with values found earlier (Section 4.3.2). Column efficiency was also pairing-ion dependent, with optimal plate heights occurring above 3×10^{-4} $mol.dm^{-3}$ pairing-ion concentration.

At a mobile phase concentration of 4×10^{-4} SDDS acceptable column efficiency was obtained, and the tryptophan was well separated from the endogenous components of the biological fluid extracts. It was found that storage of biological fluids containing tryptophan (even at $-0.11^{\circ}C$) was not possible due to the rapid degradation of tryptophan, and so biological samples were chromatographed as soon as possible after extraction.

Fig. 4.37. The relationship between pairing-ion concentration and chain length (alkylsulphate) and the capacity ratio of tryptophan. Conditions as Table 3.37. The numbers refer to the pairing-ion homologue number.



All estimations of tryptophan were made on the basis of peak height determinations and the linearity of response was checked by calibration graphs of spiked samples of plasma and saliva and aqueous solutions. Initially an internal standard of 4-aminophenol was used in aqueous buffer solutions, however this was not resolved from the endogenous peaks. Thus 4-aminophenol was replaced by a more hydrophobic internal standard, 4-chloroaniline, which eluted after tryptophan. Table 4.19 shows that the calibration graph for spiked plasma did not pass through the origin due to the presence of endogenous tryptophan. Since it is impossible to obtain tryptophan free plasma all determinations of tryptophan were made by comparison with an external standard of tryptophan prepared in aqueous buffer, containing 4-chloroaniline as an internal standard. The limit of detection was 10^{-10} moles and for the determination of tryptophan below 10^{-5} $mol.dm^{-3}$ injections of 100 µl were required. Injection of large volumes of sample resulted in band broadening such that tryptophan was not completely resolved from the internal standard, which was therefore omitted from further measurements. The coefficient of variation in the height of tryptophan was 1.68% for ten consecutive injections, allowing determinations to be made where necessary without the use of an internal standard.

The selectivity of the method was demonstrated by injecting controls of plasma, saliva and urine and by the separation of tryptophan from its four major metabolites, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindole-3-acetic acid and kynurenine Fig 4.38). No peak corresponding to tryptophan was detected in saliva. The peaks corresponding to tryptophan in urine and plasma

Table 4.19. Least squares linear regression analyses of the relationship between detector response and the spiked concentration of tryptophan in various media. The determination of detector response was made on the basis of peak height or peak height ratios.

Media	Basis of Measurement	Concentration Range ($mol.dm^{-3}$)	Slope (Standard deviation)	Intercept (Standard deviation)	Correlation Coefficient)
Plasma	Peak height	$10^{-4} - 10^{-5}$	4.85×10^{5} (5.11×10^{4})	12.6 (4.37)	0.9890
Plasma	Peak height ratio	$10^{-4} - 10^{-5}$	4.20×10^{3} (2.00×10^{2})	0.071 (0.017)	0.9977
Saliva	Peak height	$10^{-5} - 10^{-6}$	6.47×10^{6} (5.95×10^{5})	0.070 (0.034)	0.9875
Buffer	Peak height	$10^{-4} - 10^{-5}$	5.11×10^{5} (1.56×10^{4})	0.100 (0.128)	0.9986
Buffer	Peak height ratio	$10^{-4} - 10^{-5}$	5.92×10^{3} (2.55×10^{2})	0.012 (0.029)	0.9972
Buffer	Peak height	$10^{-5} - 10^{-6}$	1.30×10^{7} (6.39×10^{5})	6.64 (5.21)	0.9964

were identified by spiking with authentic samples.

In general, metabolites of biological substances are more polar than their parent compound since this facilitates urinary excretion by the kidney. The gain of a hydrophilic substituent OH reduces the capacity ratio from 4.00 to 1.5, corresponding to a τ value -0.53, which consistent with values given in Table 2.1. Loss of an uncharged COOH has less effect on retention reducing the capacity ratio from 1.5 for 5-HTP to 1.2 for 5-HT. Loss of a charged amino group in a non ion-pair system would be expected to result in an increase in retention, however in this case a decrease in retention occurs since 5-HIAA is unable to ion-pair with SDDS. Splitting the hydrophobic indole ring to give kynurenine is partly compensated for by the fact that kynurenine has two free charged amino groups and hence will be expected *a priori* to form a 2:1 complex with SDDS.

Enzymatic bioconversion does not always result in the simple addition or removal of one functional group, and the application of a functional group approach to such systems becomes difficult. As a result it is proposed here that enzyme selectivity constants, τ_e, be applied, such that:

$$\tau_e = \log (\kappa_{product} \cdot \kappa_{substrate}^{-1}) \qquad (157)$$

where $\kappa_{product}$ and $\kappa_{substrate}$ are the capacity ratios of the product and substrate respectively. Table 4.20 gives the capacity ratios of the major metabolites of tryptophan and the corresponding τ_e. Of course since τ_e is analogous to τ its value will be affected in a similar manner by changes in environmental factors.

Fig. 4.38. Separation of the main metabolites of tryptophan. Stationary phase: Spherisorb S5 ODS (Column A). Mobile phase: Methanol:water (1:1), 4 x 10^{-4} mol.dm^{-3} SDDS, pH 2.2. Flow rate: 1.5 ml.min^{-1}. Temperature: 30°C. Key : 1.5-HIAA, 2.5-HT, 3.5-HTP, 4.Kynurenine, 5. Tryptophan.

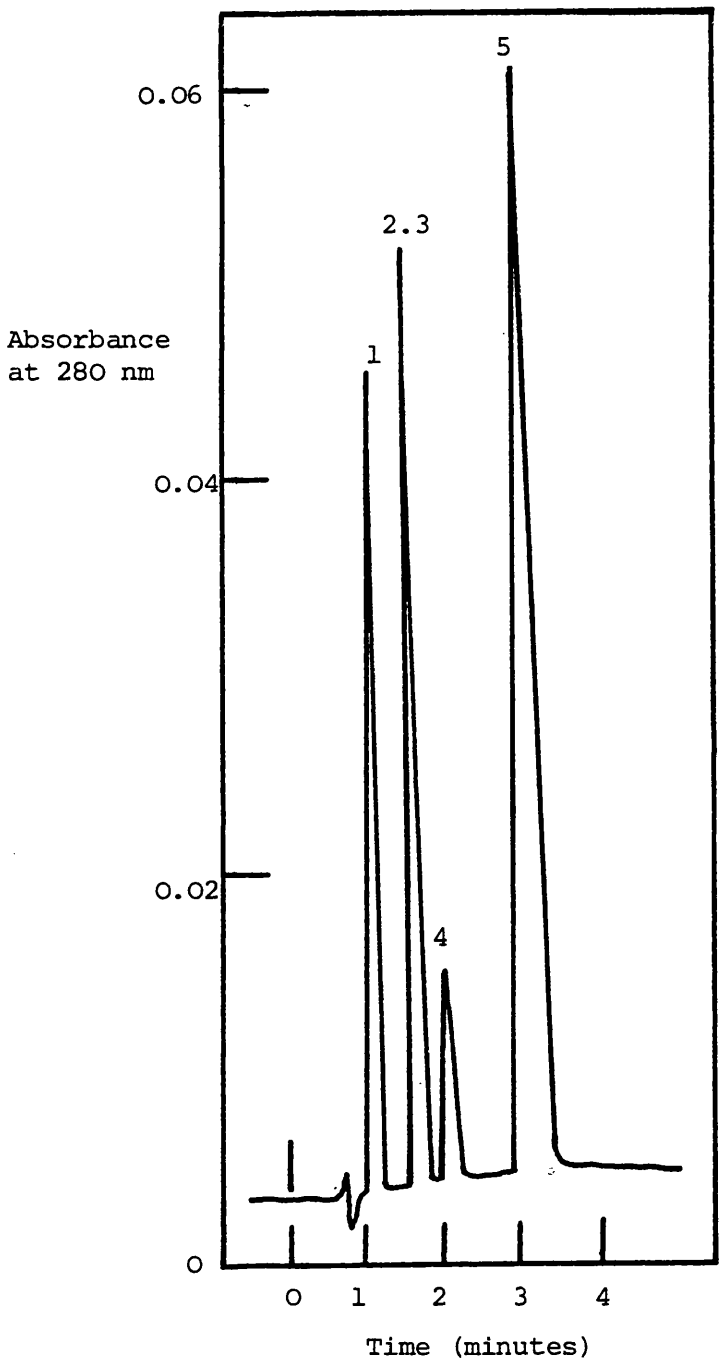
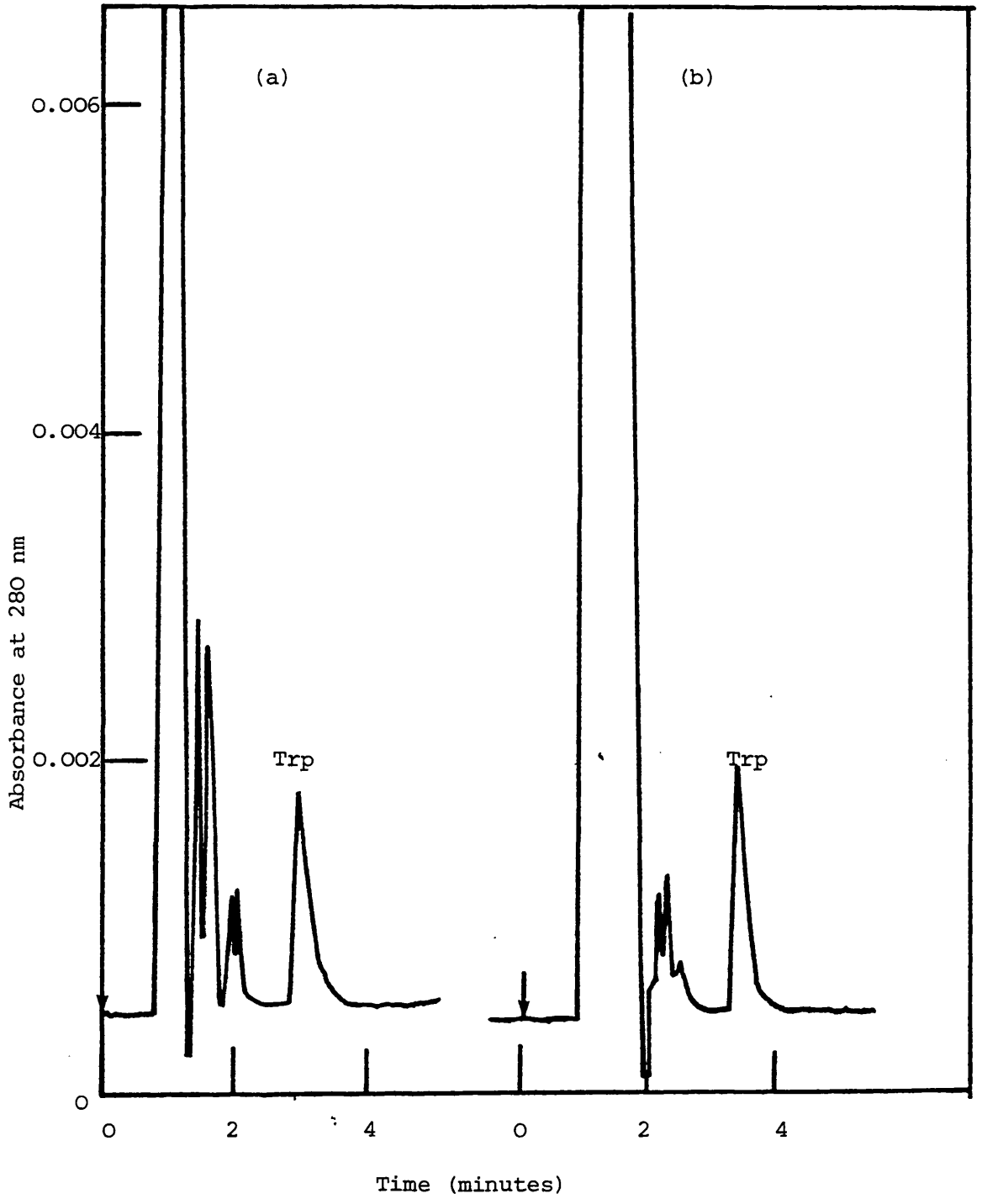


Fig. 4.39. Chromatograms of (a) saliva and (b) plasma containing 6×10^{-6} mol.dm^{-3} tryptophan. Injection volume: 100µ. Temperature ambient. Other chromatographic conditions as Fig. 4.38.



The urinary excretion profile and bioavailability of tryptophan after a single oral loading dose have been determined in fasting male subjects using the developed assay. For the urinary excretion study 1 g (i.e. 2 x 500 mg Optimax tablets) was administered orally and urine samples taken at intervals over a period of 6 hours. For the bioavailability study 2 g (i.e. 4 x 500 mg Optimax tablets) was administered, and 10 ml blood and saliva samples were taken at regular intervals over a period of 6 hours. Each saliva sample was taken over a period of 10 minutes, with no attempt made to stimulate salivation artificially.

Table 4.21 shows the concentration of tryptophan found in urine and the total amount excreted with time. It can be seen that tryptophan is excreted rapidly with about 20 mgm of the total 1 g dose excreted unchanged. The majority of the dose is excreted as the metabolites, in particular kynurenine, and it can be seen that the peak corresponding to kynurenine is considerably increased after dosage with tryptophan (Fig. 4.40). The salivary levels correlate well with plasma levels (Table 4.22) which reinforces the original hypothesis that the tryptophan in saliva is in equilibrium with the free tryptophan in plasma.

4.4.3 Sodium 4-hydroxymandelate and 6-chlorocinnoline-3-carboxylic acid

Previously, the analysis of these two solutes had proved exceptionally difficult by conventional reversed phase HPLC procedures since both eluted as broad peaks, attributed to the close proximity of the two nitrogens (CCA) and the α-hydroxyl (SHM) to the carboxyl group (248). Although these two solutes would not be present in the same samples, it was convenient for the purposes

Table 4.20. The capacity ratios of tryptophan and its metabolites and the corresponding τ_e values (Eq. 185). Stationary phase: Spherisorb S5 ODS (column B). Mobile phase: Methanol:water (1:1), 4 x 10^{-4} mol.dm^{-3} SDDS, pH 2.25 (citrate buffer). Ambient (18-22°C).

Solute	κ	log τ_e	Enzyme
Tryptophan	4.00*	-	-
Kynurenine	2.2	-0.25	Pyrrolase
5-HTP	1.5	-0.53	Hydroxylase
5-HT	1.2	-0.11	Carboxylase
5-HIAA	0.5	-0.48	Mono-amine oxidase

*n.b. the slightly higher value for the capacity ratio of tryptophan is due to the lower operating temperature (ambient c.f. 30°C).

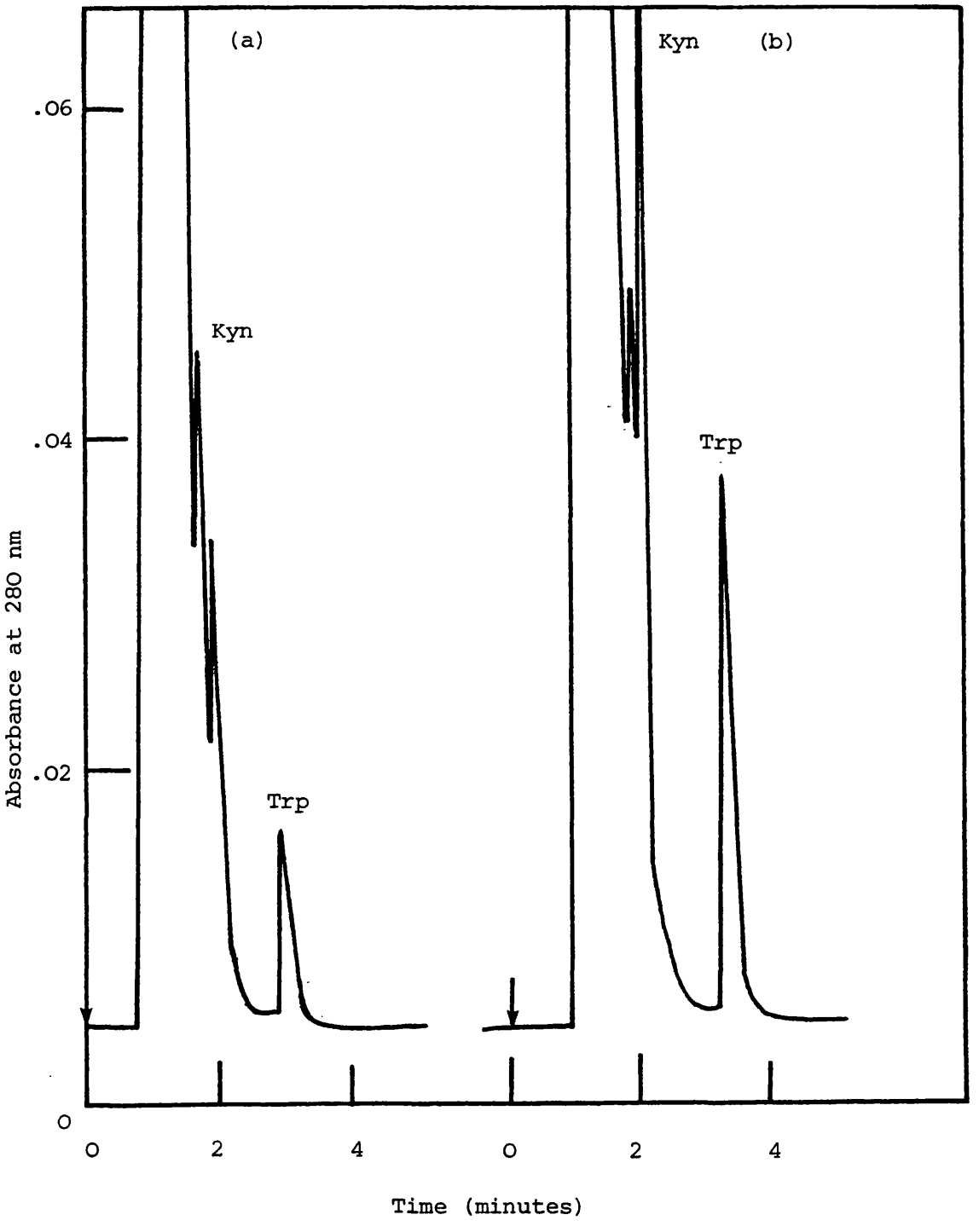
Table 4.21. The concentration of tryptophan and the total amount of tryptophan excreted in urine with time after a 1 g oral dose.

Time (h)	Tryptophan concentration in urine $(mol.dm^{-3}) \times 10^{4}$	($\mu g\ ml^{-1}$)	Volume of urine taken (ml)	Cumulative amounts of tryptophan excreted (moles)$\times 10^{5}$	(mg)
0	1.38	27.9	90	1.24	2.51
0.62	1.22	24.7	80	2.22	4.49
1.55	3.64	73.6	54	4.18	8.45
2.87	1.74	35.2	195	7.58	15.33
5.42	2.09	42.3	71	9.67	19.55

Table 4.22. Concentration of free tryptophan in plasma and saliva with time after a 2g. oral dose.

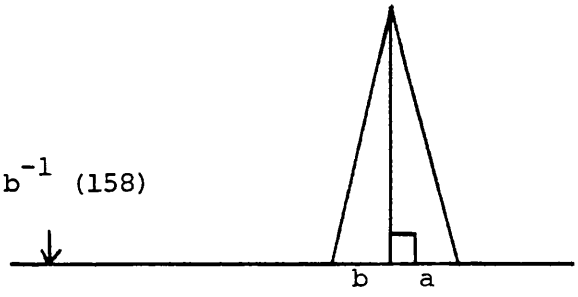
Time (h)	Plasma concentration $(mol.dm^{-3}) \times 10^{5}$	Plasma concentration $(\mu g\ ml^{-1})$	Time (h)	Saliva concentration $(mol.dm^{-3}) \times 10^{5}$	Saliva concentration $(\mu g\ ml^{-1})$
0	0.78	1.57	0	0.14	0.28
0.58	1.49	3.02	0.92	0.19	0.38
1.08	2.23	4.51	1.72	0.28	0.57
1.70	2.82	5.71	3.42	0.22	0.44
4.58	1.06	2.14	4.78	0.13	0.27
5.58	0.59	1.19			

Fig. 4.40. Chromatograms of urine before (a) and 16 hours after oral administration of 1 g of tryptophan (b), showing increased levels of excreted tryptophan and kynurenine. Conditions as Fig. 4.38.



of routine analysis to develop an assay procedure applicable to both solutes. Figure 4.41 shows the relationship between capacity for these solutes and the C_{14}BDAC in the mobile phase of 30% v/v acetonitrile at pH 7.5. It is demonstrated that optimal analytical conditions were achieved with a mobile phase containing 5×10^{-4} mol.dm^{-3} C_{14}BDAC at which the capacity ratios of CCA and SHM were 3.92 and 0.74 respectively. Benzoic acid eluted between the two solutes of interest (Fig. 4.43) and was used as an internal standard. To investigate the kinetics of retention of these solutes, each was injected six times and the number of theoretical plates (Eq. 2) and the peak symmetry (Eq. 158) calculated.

$$\text{Peak Symmetry} = a.b^{-1} \quad (158)$$

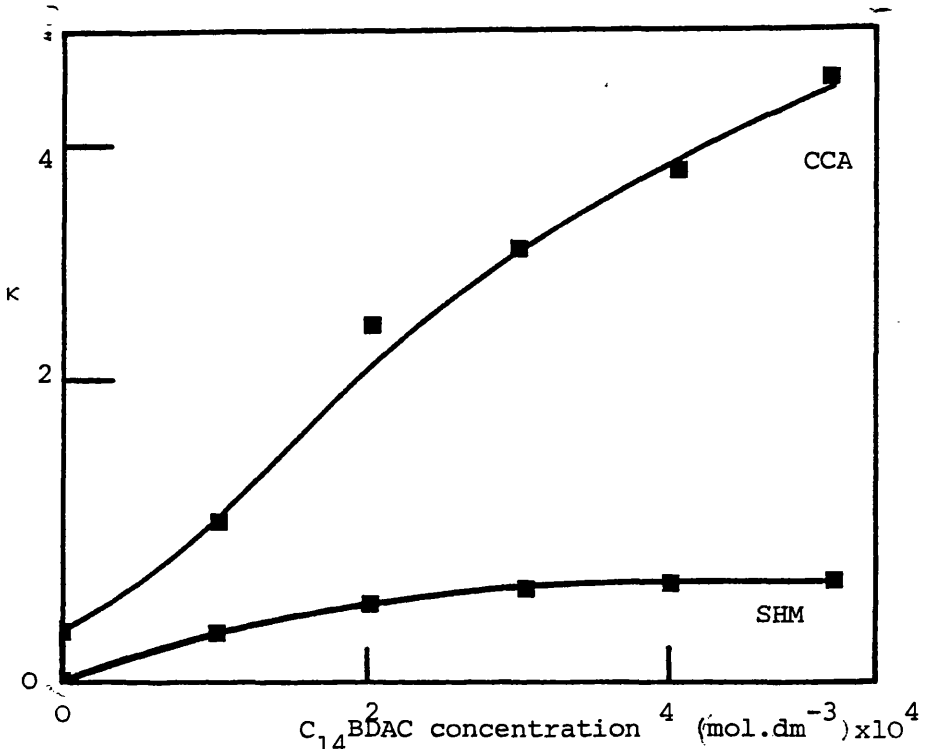


The column efficiencies ranged between 1020 (SHM) and 1606 (CCA) and there was no statistical difference between the three peaks at the 99% level. All the peaks were slightly tailed due to the injection system employed (loop valve) and peak symmetry improved with retention, with the symmetry of CCA better than both SHM at the 99% level and better than BA at the 95% level.

The peak heights and peak height ratios were linear with respect to concentration (Fig. 4.43 and Table 4.23) and the coefficients of variation of peak heights were 1.49% for SHM and 2.80% for CCA, whereas the coefficients of variation of peak height ratios were 0.90% for SHM and 0.69% for CCA (n = 6, in each case). In this present study it has been observed in reversed phase

Fig. 4.41. κ *versus* pairing-ion concentration (C_{14}BDAC) for SHM and CCA. Conditions as Tables 3.47 and 3.48.

Fig. 4.42. Peak height ratios for CCA and SHM *versus* concentration injected. Internal standard benzoic acid (0.1 mg.ml^{-1}). Conditions as Fig. 4.43.



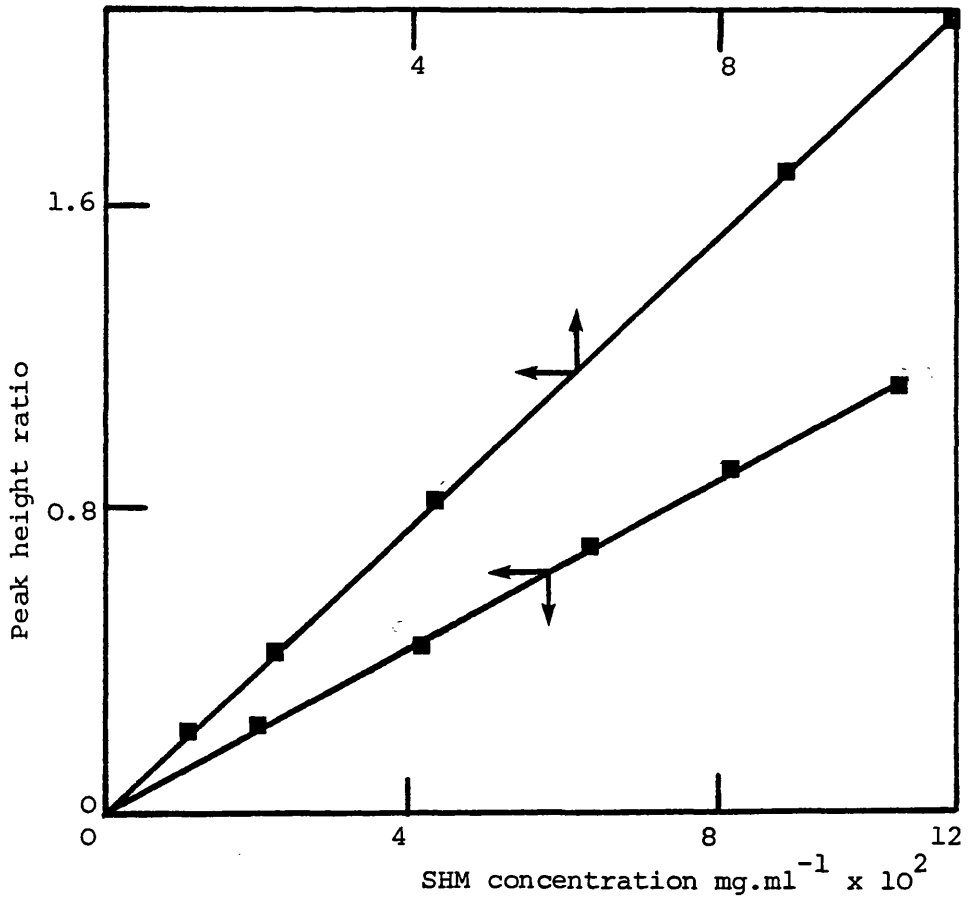


Fig. 4.43. Separation of 6-chlorocinnoline-3-carboxylic acid (CCA), sodium 4-hydroxymandelate (SHM) and benzoic acid (BA). Stationary phase: ODS Hypersil (Column G). Mobile phase: 30% v/v acetonitrile, 5×10^{-4} C_{14}BDAC, pH 7.5. Temp. 30°C.

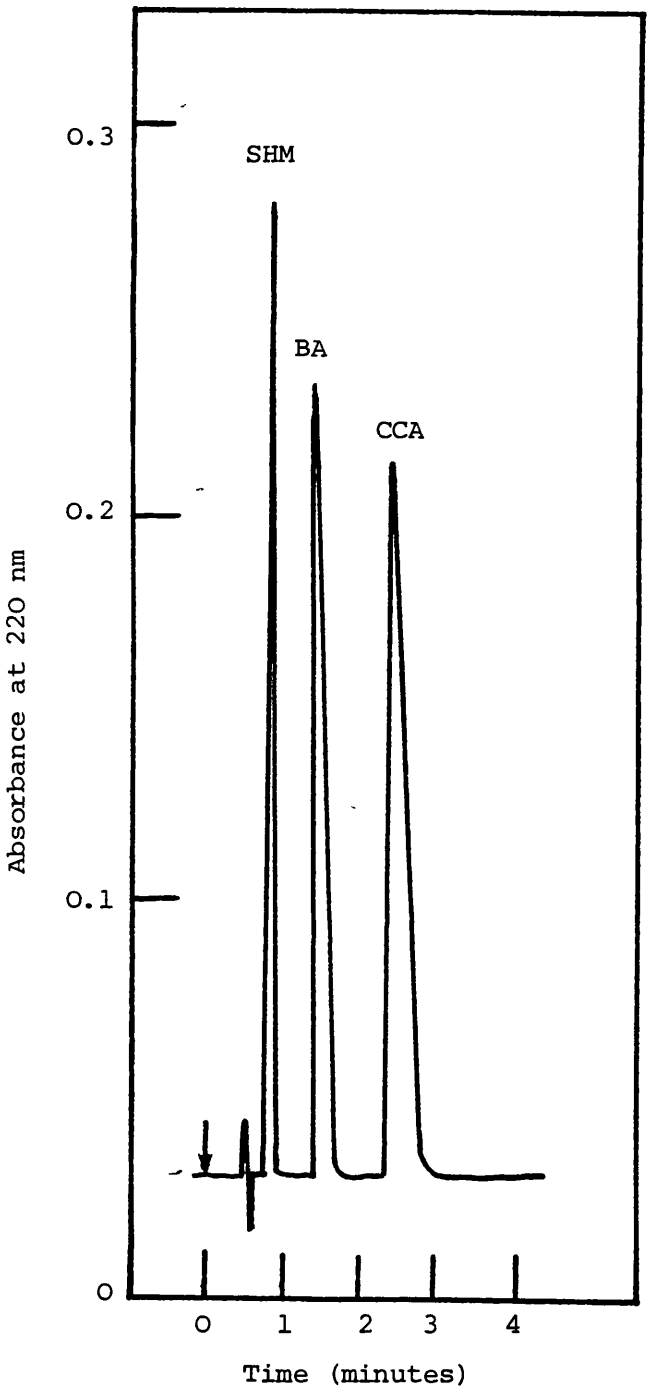


Table 4.23. Least squares linear regression analysis of the relationships between peak heights and peak height ratios of CCA and SHM and concentration injected. Table 3.47 and Table 3.48.

Solute	Basis of Measurement	Concentration Range $mg.ml^{-1}$	Slope (standard deviation)	Intercept (standard deviation)	Correlation Coefficient
CCA	Peak height	0.0021-0.0103	1.27×10^4 (7.12×10^2)	3.38 (4.86)	0.995
CCA	Peak height ratio	0.0021-0.0103	18.7 (0.21)	0.016 (0.014)	0.999
SHM	Peak height	0.011-0.110	1.95×10^3 (37.1)	3.32 (2.48)	0.999
SHM	Peak height ratio	0.011-0.110	18.8 (0.207)	0.014 (0.014)	0.999

ion-pair systems that while steric and intramolecular effects influence the thermodynamics of transfer, the kinetics are unaffected. As a consequence the addition of a bulky hydrophobic pairing-ion (C_{14}BDAC) to an aqueous acetonitrile mobile phase shielded the carboxyl group and the two solutes eluted as narrow almost symmetrical bands.

The work of Knox and Laird (120) demonstrated the improved kinetics of transfer of ionised solutes in reversed phase ion-pair systems using surfactant pairing-ions compared with straight phase systems. In this study the advantages of these systems compared with non ion-pair systems where intramolecular effects can produce poor peak shape is demonstrated.

4.4.4 Structural isomers of hydroxyphenylglycine.

Commercial samples of 4-hydroxyphenylglycine generally contain small amounts of the 2-isomer and previous attempts to separate these two had proved unsuccessful using adsorption HPLC methods (248). We have shown previously (Section 4.3.7) that reversed phase ion-pair techniques employing an acidic mobile phase containing SDDS (5×10^{-4} mol.dm^{-3}) are suitable for the separation of amino-acids. Additionally, the resolution of 2- and 4-isomers is found to be greatly influenced by the concentration and type of organic modifier in the mobile phase (Section 4.3.3).

The effect of methanol and acetonitrile at a fixed pairing-ion concentration (5×10^{-4} mol.dm^{-3} SDDS) on retention and steric selectivity have been investigated. Use has been made of a steric selectivity factor, τ_s, where:

$$\tau_s = \log (\kappa_2 . \kappa_4^{-1}) \qquad (159)$$

Table 4.24. The capacity ratios of 4-hydroxyphenylglycine and 2-hydroxyphenylglycine and the steric selectivity factor, τ_s, (Eq. 159) with various organic modifier compositions. Stationary phase: ODS Hypersil (Column G), Mobile phase: Methanol or acetonitrile, 5×10^{-4} $mol.dm^{-3}$ SDDS, pH 2.2 (adjusted with H_3PO_4), 30°C.

Organic modifier		k		
Type	Concentration (% v/v)	2-HPG	4-HPG	τ_s
Methanol	25	27.5	7.50	0.56
Methanol	40	9.66	5.00	0.29
Methanol	50	3.47	2.10	0.21
Acetonitrile	20	4.67	1.23	0.58

Fig. 4.44. Chromatogram of 4-hydroxyphenylglycine showing the presence of 0.54 w/w 2-hydroxyphenylglycine. Stationary phase: ODS Hypersil (Column G). Mobile phase: 20% v/v acetonitrile, 5×10^{-4} mol.dm^{-3} SDDS, pH 2.2. 30°C.

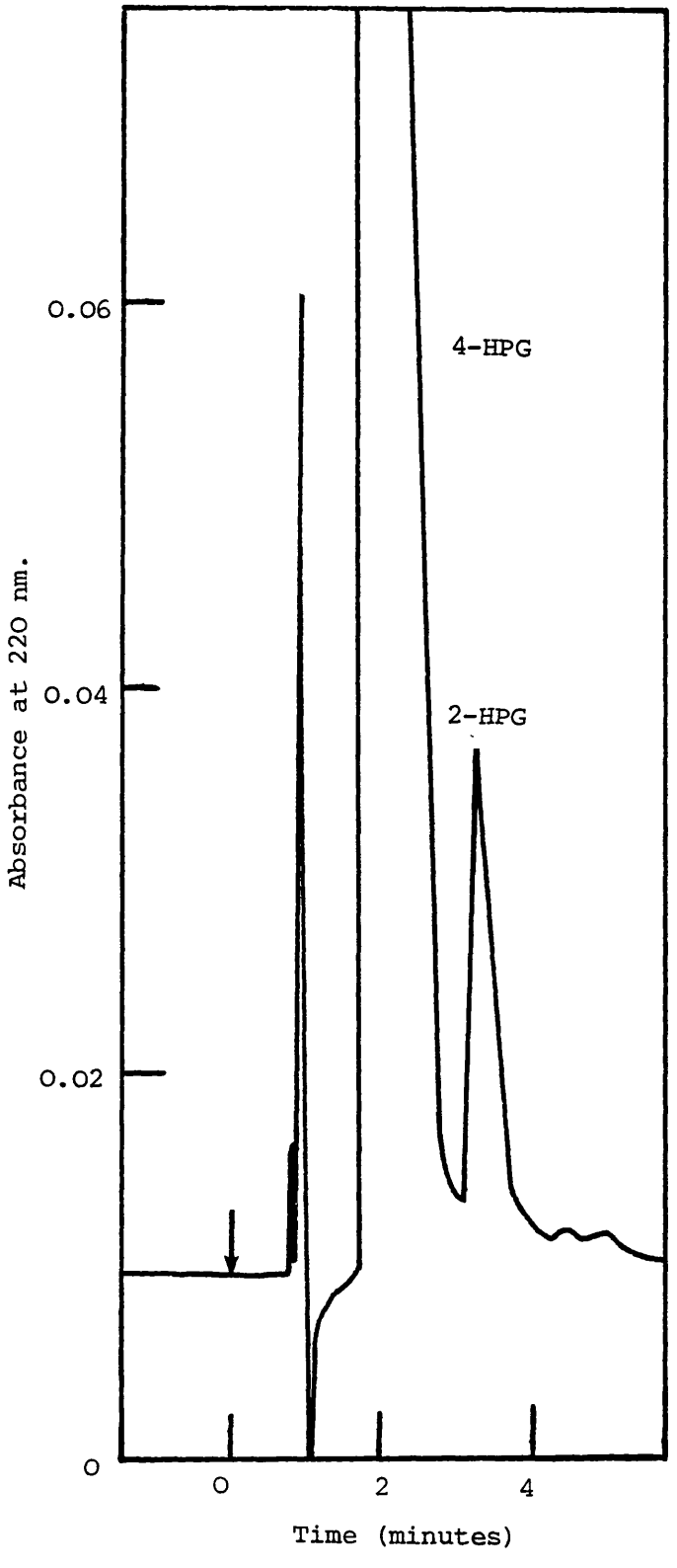
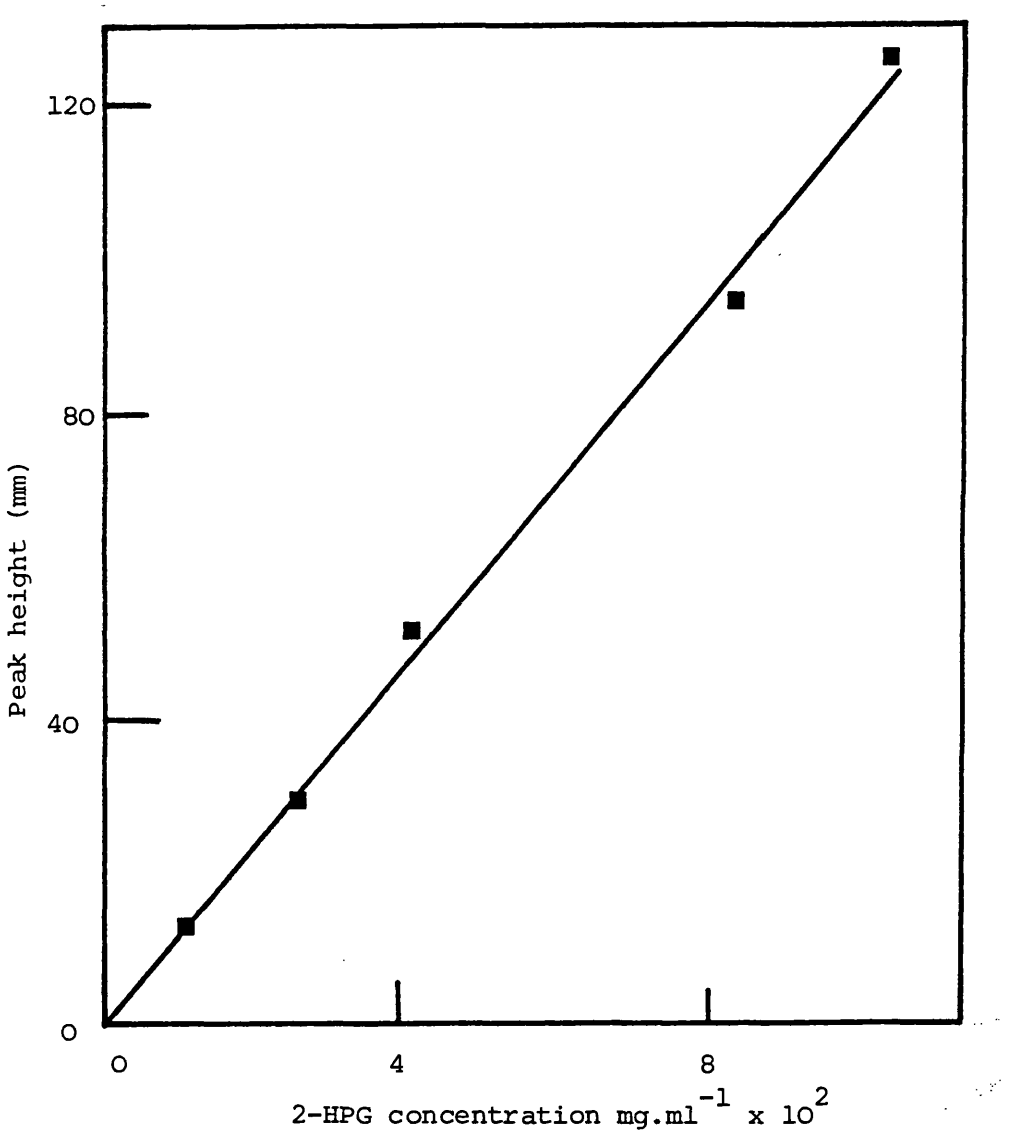


Fig. 4.45. Relationship between the peak height of 2-hydroxyphenylglycine (2-HPG) and concentration injected. Table 3.49. Chromatographic conditions as Fig. 4.44.



Initial experiments showed that increasing the methanol concentration from 25% v/v to 50% v/v decreased both retention and the value of τ_s. (50% v/v methanol gave satisfactory capacity ratios for the two isomers however the selectivity factor (τ_s = 0.21) was too small for adequate resolution). It was then found (Table 4.24) that although 20% v/v acetonitrile gave an equivalent separation of the two isomers, the steric selectivity factor as 0.58 which permitted adequate resolution in that trace amounts of 2-hydroxyphenylglycine could be determined. Figure 4.44 shows a chromatogram of 4-hydroxyphenylglycine containing 0.54% w/w of the 2-isomer.

The peak height of 2-hydroxyphenylglycine was linear with respect to concentration over the range 0.01 to 0.10 $mg.ml^{-1}$ (n = 5, r = 0.999) (Fig. 4.45) and the coefficient of variation of peak height was 2.01% (n = 8).

4.4.5 Beta-adrenergic receptor blockers and related compounds.

Atenolol (I) is a weak base, ionised at low pH, and was required to be separated from five structurally related analogues (II to VI) shown in Fig. 2.2. IV, V and VI are neutral and their retention in reversed phase systems is expected to be controlled by the concentration and type of the organic modifier in the mobile phase. Atenolol, II and III are cationic and their retention at low pH will be controlled not only by the organic modifier but also by the concentration and type of pairing-ion in the mobile phase. The physicochemical properties responsible for selectivity are both hydrophobic (e.g. IV cf. V) and polar (e.g. II, cf. I and III).

Initially the effect of two organic modifiers at equivalent concentrations (Fig. 4.46) were compared at pH 2.0 in the absence of a pairing-ion: i.e. methanol (30% v/v) and acetonitrile (16% v/v). These two mobile phases gave the same retention for the neutural solute, VI, however the selectivities for the separations of the three neutral solutes (IV, V and VI) were significantly different (Fig. 4.46). This may be rationalised in terms of the different solute-solvent interactions between the two organic modifiers and the polar functional groups of the neutral solutes (Section 4.4.3) - namely the epoxide group in IV and the additional amide group in V.

In the absence of a pairing-ion the ionised solutes eluted relatively quickly, however there were noticeable differences in selectivity within this group, with II and III being much better resolved in the acetonitrile phase system compared with the methanol system. Atenolol gave the same retention in both phase systems. Addition of a pairing-ion (SOS) enhanced the retention of the ionised solutes and left the neutral solutes relatively unaffected (Fig. 4.48). However the selectivity coefficients (τ values) for the ionised solutes were independent of pairing-ion concentration (Table 4.25) confirming previous results (Section 4.3.1). Due to the different selectivities of the organic modifiers, optimal separation was achieved at different pairing-ion concentrations - namely 2.5×10^{-4} mol.dm^{-3} SOS in acetonitrile (16% v/v), and 7.5×10^{-3} SOS in methanol (30% v/v). Due to the greater selectivity with respect to the separation of II and III, the acetonitrile systems was preferred (Fig. 4.47).

Fig. 4.46. Separation of the three neutral solutes (IV, V and VI) related to atenolol, showing the different selectivities of methanol (30% v/v) and acetonitrile (16% v/v). Stationary phase: ODS Hypersil (Column G). Mobile phase: 0.1% v/v H_2SO_4 containing either methanol or acetonitrile Temperature: 30°C

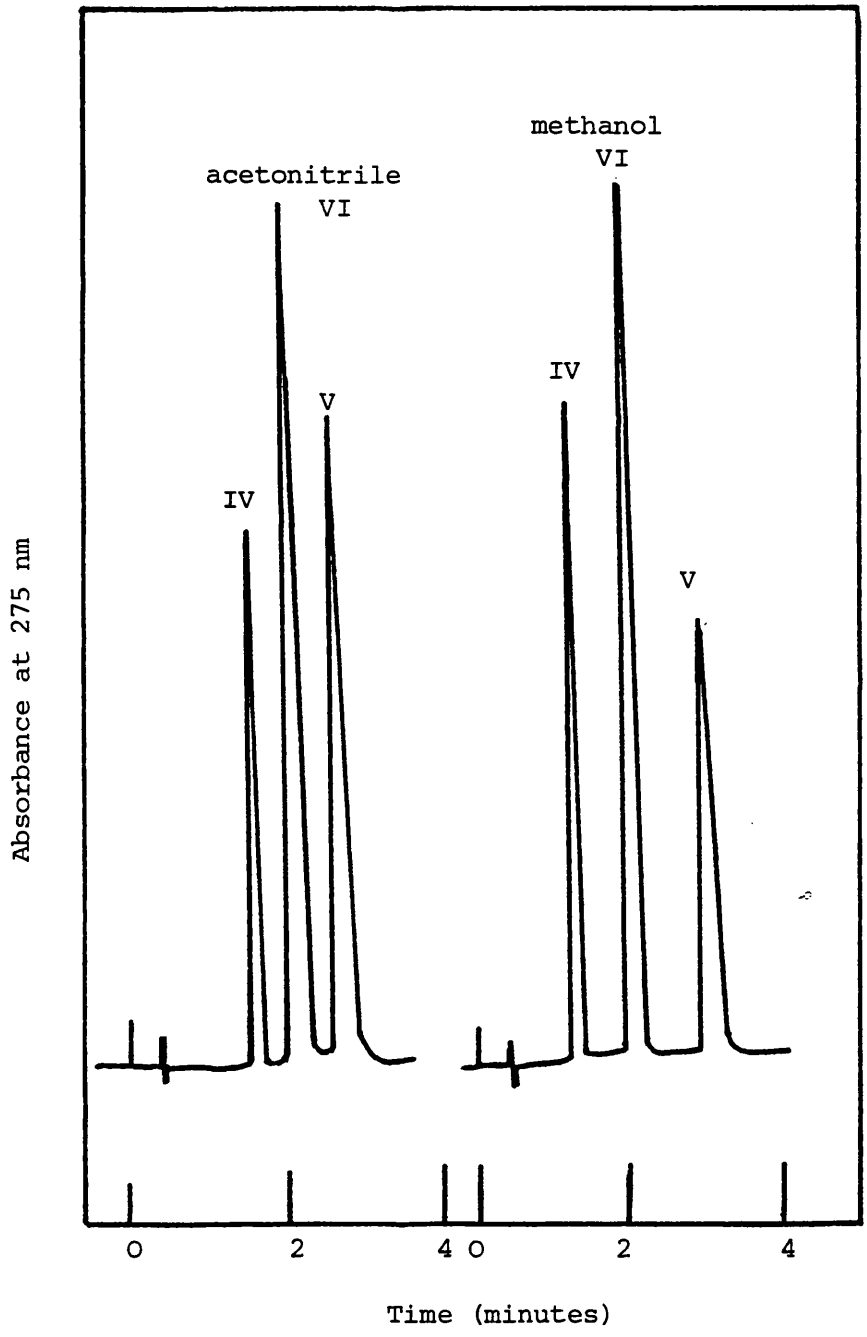


Fig. 4.47. Separations of atenolol related compounds. Stationary phase: ODS Hypersil (Column G). Mobile phases: (a) 16% v/v acetonitrile, 2.5 x 10^{-4} mol.dm^{-3} SOS, pH 2.2 (b) 30% v/v methanol, 7.5 x 10^{-4} mol.dm^{-3} SOS, pH 2.0. Temperature 30°C. Key as Fig. 2.2.

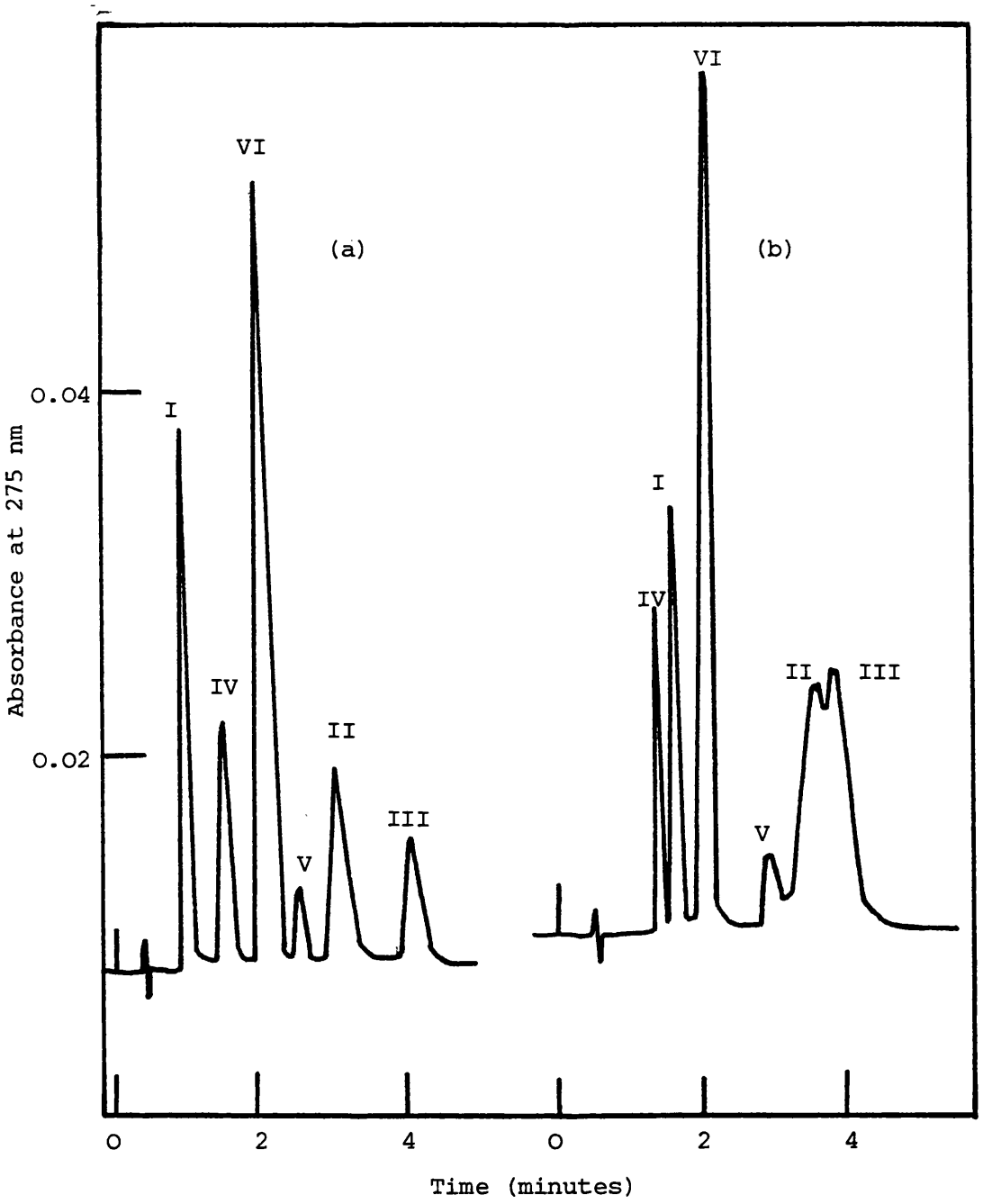
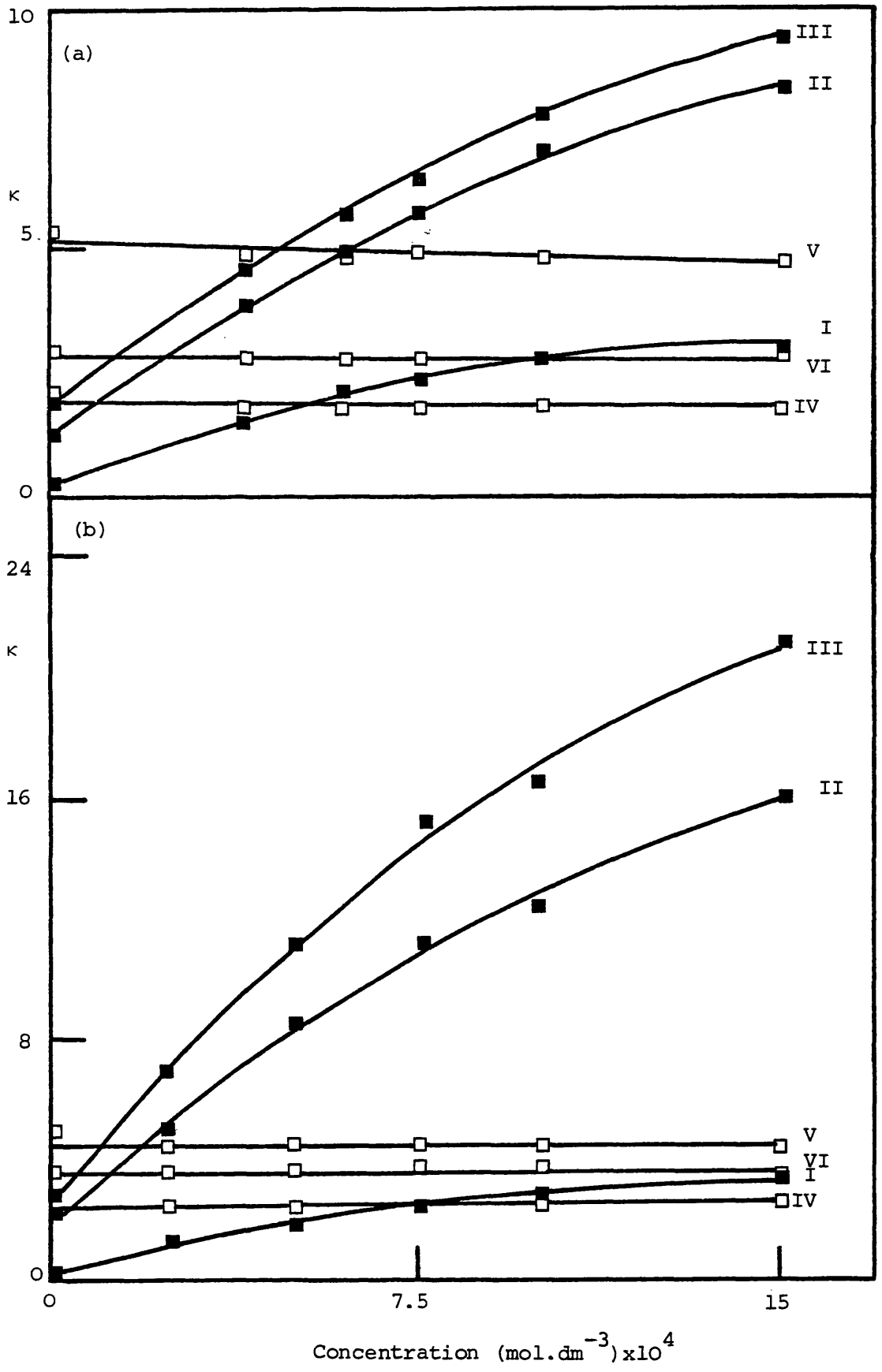


Fig. 4.48. The relationship between the capacity ratios, κ, of atenolol and related compounds and pairing-ion concentration (SOS) in mobile phases containing (a) methanol (30% v/v) or (b) acetonitrile (16% v/v). Conditions as Table 3.38. Key as Fig. 2.2.



The τ values obtained in this study on atenolol should prove useful in the prediction of the retention behaviour of compounds related to other beta-blockers. To confirm this hypothesis, τ values have been used to predict the retention of compounds related to the drug practolol, i.e. II_1 and V_1 (Fig. 2.2), at different SOS concentrations in 16% v/v acetonitrile at pH 2.0. Practolol, II_1 and V_1 are isomers of atenolol, II and V respectively and if the capacity ratio of practolol (κ_{I_1}) is known then κ_{II_1} and κ_{V_1} may be calculated from:

$$\log \kappa_{II_1} = \log \kappa_{I_1} + \tau_{II} \quad (160)$$

$$\log \kappa_{V_1} = \log \kappa_{I_1} + \tau_{V} \quad (161)$$

Since II and V are dimeric forms of atenolol, the τ values depend on the beta-blocker series from which they were determined. Thus for dimeric practolol analogues the capacity ratios are given by:

$$\log \kappa_{II_1} = \log \kappa_{I_1} + \tau_{II} + \tau_{I_1:I} \quad (162)$$

and

$$\log \kappa_{V_1} = \log \kappa_{I_1} + \tau_{V} + \tau_{I_1:I} \quad (163)$$

where

$$\tau_{I_1:I} = \log (\kappa_{I_1} . \kappa_{I}^{-1}) \quad (164)$$

Here for the general case relationships for beta-blocker, Z, analogues, ZX are:

Monomers

$$\log \kappa_{ZX} = \log \kappa_{Z} + \tau_{X} \quad (165)$$

Dimers

$$\log \kappa_{ZX} = \log \kappa_{Z} + \tau_{X} + \tau_{Z:I} \quad (166)$$

Table 4.26 gives the capacity ratios of practolol and the corresponding $\tau_{I_1:I}$ values (Eq. 164) at different SOS concentrations in 16% v/v acetonitrile at pH 2.0. Table 4.27 and Fig.4.49 shows the observed and calculated capacity ratios of the practolol analogues II_1 and V_1, at different SOS concentrations. The agreement between the observed and calculated capacity ratios for II_1 and V_1 gives confidence in the suggestion that the τ values based on the atenolol series may be used to predict the retention of other beta-blocker analogues.

Table 4.25. τ values of some atenolol analogues(II to VI) at different pairing-ion concentrations (SOS). Stationary phase: ODS Hypersil (Column G). Mobile phase: 16% v/v acetonitrile, pH 2.0 (0.1% H_2SO_4). 30°C.

Analogue	τ					
	SOS concentration (mol.dm^{-3})x10^3					
	0.00	0.25	0.50	0.75	1.00	1.50
II	0.66	0.63	0.63	0.60	0.64	0.63
III	0.73	0.76	0.74	0.73	0.75	0.75
IV	0.73	0.12	0.08	-0.07	-0.09	-0.20
V	1.03	0.67	0.37	0.20	0.22	0.10
VI	0.89	0.27	0.16	0.07	0.09	-0.04

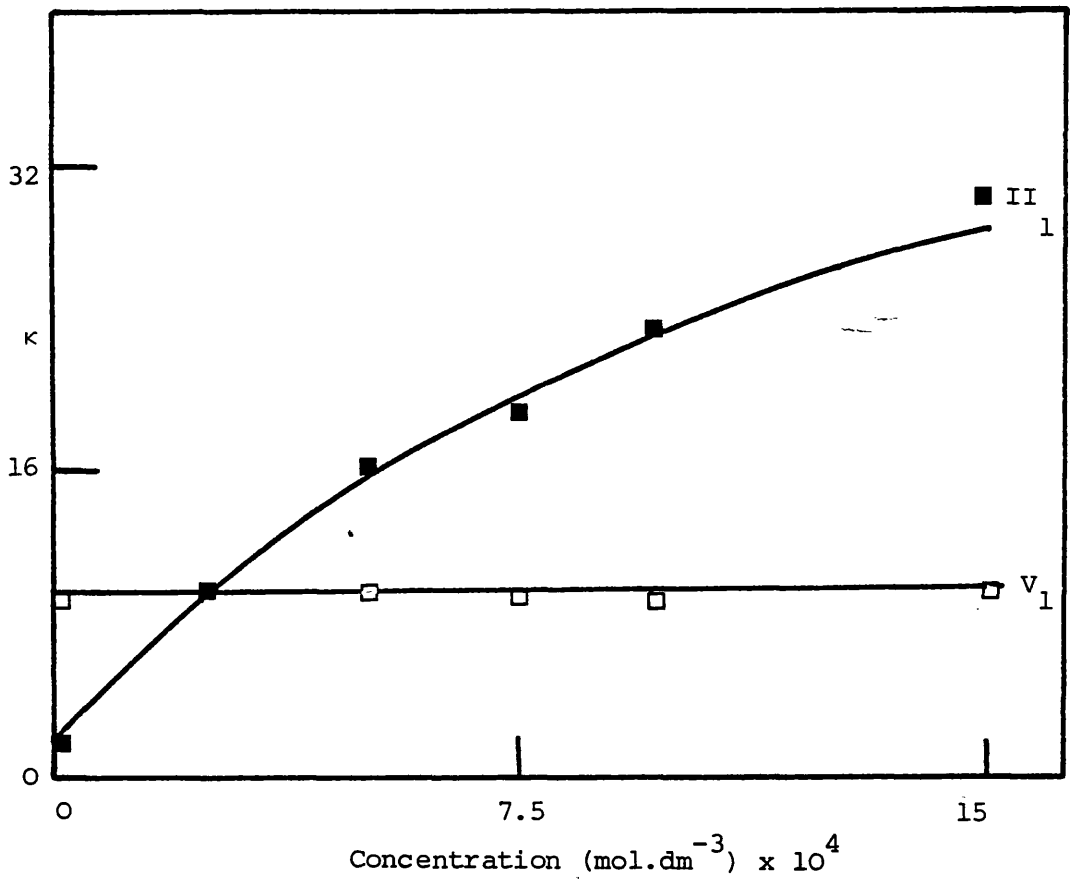
Table 4.26. The capacity ratios of practolol and the corresponding $\tau_{I_1:I}$ values (Eq. 164) at different pairing-ion concentrations (SOS). Stationary phase: ODS Hypersil (Column G). Mobile phase: 16% v/v acetonitrile. pH 2.0 (0.1% H_2SO_4). 30°C.

SOS concentration (mol.dm^{-3})x10^3	κ_{I_1}	$\tau_{I_1:I}$
0.00	0.68	0.16
0.25	1.81	0.14
0.50	2.69	0.12
0.75	3.86	0.13
1.00	3.84	0.14
1.50	5.57	0.17

Table 4.27. The observed and calculated capacity ratios of the analogues of practolol (II_1 and V_1) at different pairing-ion (SOS) concentrations. Stationary phase: ODS Hypersil (Column G). Mobile phase: 16% v/v acetonitrile pH 2.0. 30°C.

SOS concentration $(mol.dm^{-3}) \times 10^3$	k			
	II_1		V_1	
	obs.	calc.	obs.	calc.
0.00	3.63	4.07	9.33	10.0
0.25	10.7	11.5	8.51	10.7
0.50	16.6	15.9	8.91	8.71
0.75	21.9	21.4	8.51	8.51
1.00	24.0	23.4	8.71	8.91
1.50	32.4	30.2	8.71	9.77

Fig. 4.49. Observed and predicted capacity ratios, κ, of two practolol related compounds *versus* pairing-ion (SOS) concentration. Table 4.27. Square symbols observed values, solid line predicted values.



4.4.6 Beta-adrenergic receptor blocker formulations.

a. 'Tenoretic' Tablets

'Tenoretic' is a tablet formulation containing 100 mg of atenolol and 25 mg of the diuretic chlorthalidone. An assay procedure was required which would separate the two active components of the formulation, and 2-(4-chloro-3-sulphamoylbenzoyl)-benzoic acid which is a known breakdown product of chlorthalidone, and three compounds related to atenolol namely 4-hydroxyphenylacetic acid, 4-hydroxyphenylacetamide and 4-(2'-hydroxy-3'-isopropyl-aminopropoxy)-phenylacetic acid. Previously the active components of 'Tenoretic' had been assayed separately by two straight phase HPLC methods however the previous success of the reversed phase ion-pair separation of atenolol (Section 4.4.5) suggested that it could be applied to the analysis of 'Tenoretic'.

With a stationary phase of ODS Hypersil (Column H) and a mobile phase of 16% v/v acetonitrile and 2.5 x 10^{-4} mol.dm^{-3} SOS a satisfactory capacity ratio (1.38) for atenolol resulted. However chlorthalidone was too retained (κ = 9.21). The slightly higher retention for atenolol obtained here compared with previous results (Section 4.4.5) was due to the lowered operating temperature (ambient, cf. 30°C). Optimal retention of chlorthalidone was achieved by increasing the acetonitrile concentration through 20% v/v to 25% v/v since it is known (Section 4.3.3) that an increase in modifier concentration will decrease retention. But under these latter conditions atenolol eluted close to the solvent front. Since chlorthalidone is unionised at the pH of the mobile phase the retention of the cationic atenolol was selectively increased by increasing the concentration of the pairing-ion. At a pairing-

ion concentration of 2.00 x 10^{-3} mol.dm^{-3} although atenolol was again significantly retained (κ = 1.00), this mobile phase was not selective between atenolol and 4-hydroxyphenylacetic acid, which were only partially resolved, and between chlorthalidone and 4-(2'-hydroxy-3'-isopropylaminopropoxy)-phenylacetic acid which were almost unseparated. Since only atenolol and 4-(2'-hydroxy-3'-isopropylaminopropoxy)-phenylacetic acid are ionised optimal separation conditions are thus achieved by a further increase in the pairing-ion concentration to 4 x 10^{-3} mol.dm^{-3} SOS, at which point baseline seapration of all six components is obtained. Sample chromatograms illustrating the rational optimisation of the separation are shown in Figs. 4.50 to 4.52 and demonstrate the flexibility of reversed phase ion-pair systems in separating neutral and ionised solutes.

Quantitative measurements of the chlorthalidone and atenolol were made on the basis of peak areas determined by the use of a Hewlett-Packard laboratory computer. A Varian auto-injector was used and the low coefficients of variation of peak areas (atenolol 0.19% and chlorthalidone 0.38% for n = 10) indicated that an internal standard was not necessary. The peak areas were found to be linear with respect to concentration over the ranges 0.21 to 2.06 mgm.ml^{-1} atenolol and 0.051 to 0.51 mgm.ml^{-1} chlorthalidone with correlation coefficients of 0.999 for both solutes (Fig. 4.53).

The total reliability of the procedure was shown by assaying ten samples of 'Tenoretic'. The active ingredients were determined by means of Eq. 101 (Section 2.3.8c) with reference to an external

standard containing 100 mg atenolol and 25 mg chlorthalidone in 100 ml. The results were expressed in terms of weight per tablet and concentration (w/w), and are shown in Table 4.28.

The procedure was also applied to the analysis of 'Tenormin'* injections which contain 10 mg $.ml^{-1}$ atenolol. The injections were diluted 1 in 10 in distilled water and assayed by reference to an external standard containing 1.0 $mg.ml^{-1}$ atenolol (see Fig. 4.54).

b. 'Inderetic' capsules

'Inderetic' is a capsule formulation contaiing 80 mg propranolol, a beta-blocker and 2.5 mg bendrofluazide, a diuretic. An assay procedure was required which would separate the active ingredients propranolol and bendrofluazide and their respective degradation products 1-naphthol and 5-trifluoromethyl-2,4-disulphonamidoaniline (free base). Although the British Pharmacoepia sets a limit of 2000 ppm for 1-naphthol in propranolol in practice propranolol is highly stable and has no other known degradation products. However bendrofluazide is highly unstable and the British Pharmacoepia sets a limit of 1% of free base in bendrofluazide which is determined by thin layer chromatography. In fact bendrofluazide is so unstable that previously it had to be hydrolysed to the free base by the action of sodium hydroxide before analysis.

Initial experiments revealed that the four components to be separated were considerably more solvophobic than those encountered in the 'Tenoretic' system, and consequently they were not eluted from

* 'Tenormin' is a registered trade mark of Imperial Chemical Industries Limited.

Fig. 4.50. Chromatograms of atenolol and chlorthalidone, showing the effect of increasing acetonitrile concentration.
Stationary phase: ODS Hypersil (Column H). Mobile phase: 0.1% v/v H_2SO_4, acetonitrile, 2.5 x 10^{-4} SOS. Temperature ambient. Key as Fig. 2.2.

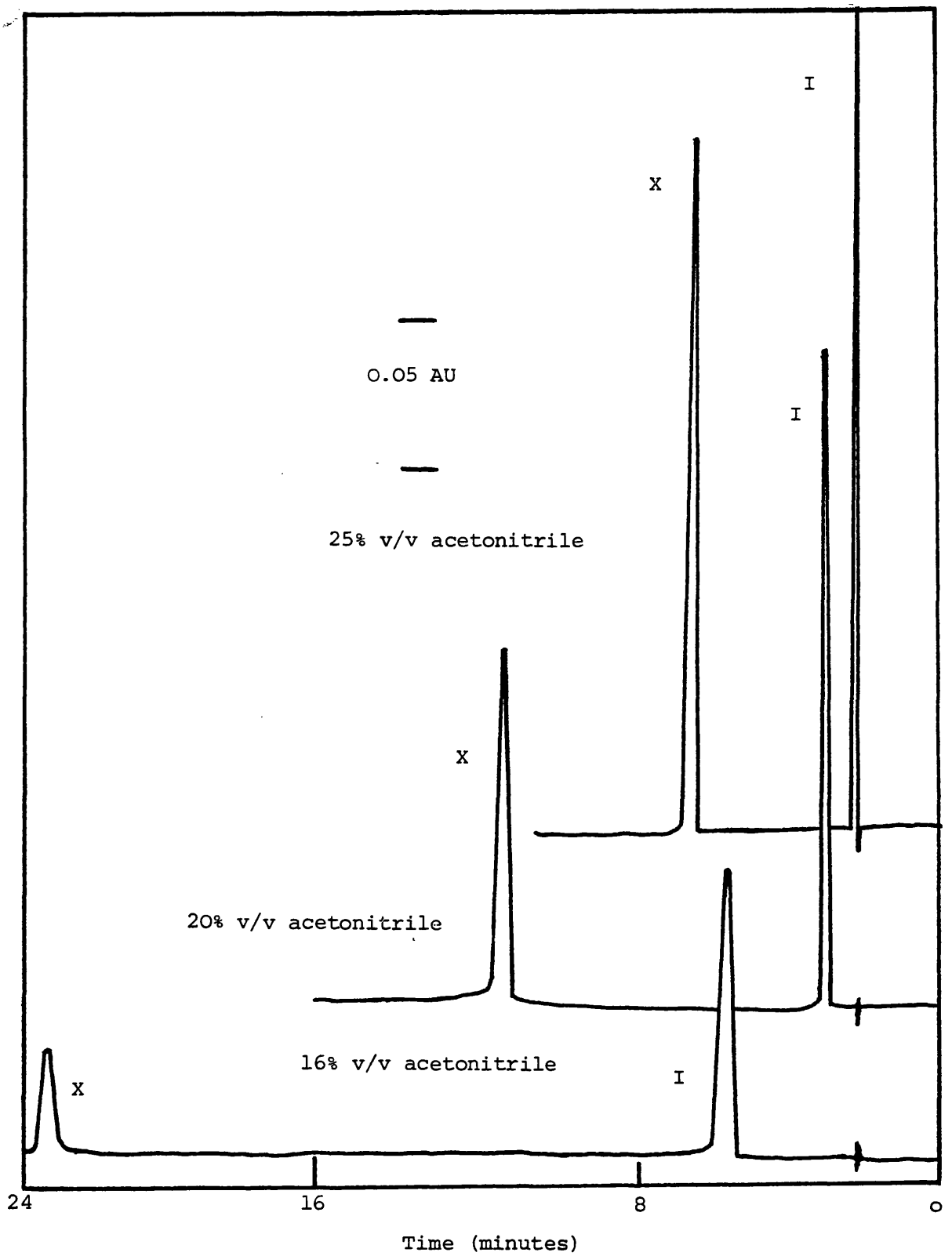


Fig. 4.51. Separations of atenolol, chlorthalidone and related compounds showing the effect of increasing the pairing-ion (SOS) concentration. Stationary phase: ODS Hypersil (Column H). Mobile phase: 0.1% v/v H_2SO_4, 25% v/v acetonitrile. Temperature: ambient. Key as Fig. 2.2.

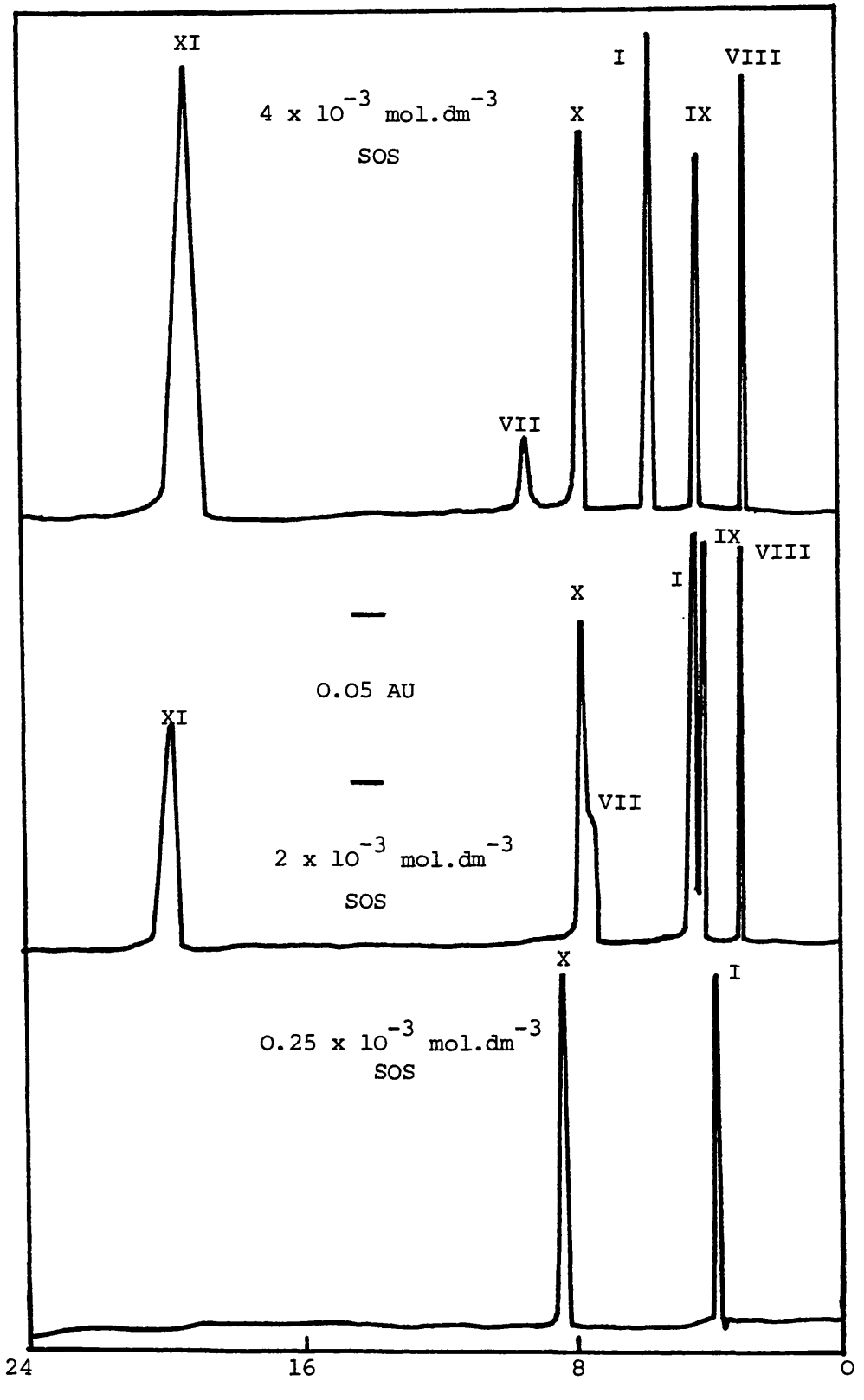


Fig. 4.52. Optimal separation of atenolol, chlorthalidone and related compounds. Stationary phase: ODS Hypersil (Column H). Mobile phase: 25. v/v acetonitrile, 0.1% v/v H_2SO_4, 4 x 10^{-3} $mol.dm^{-3}$ SOS. Temperature: ambient. Key as Fig. 2.2.

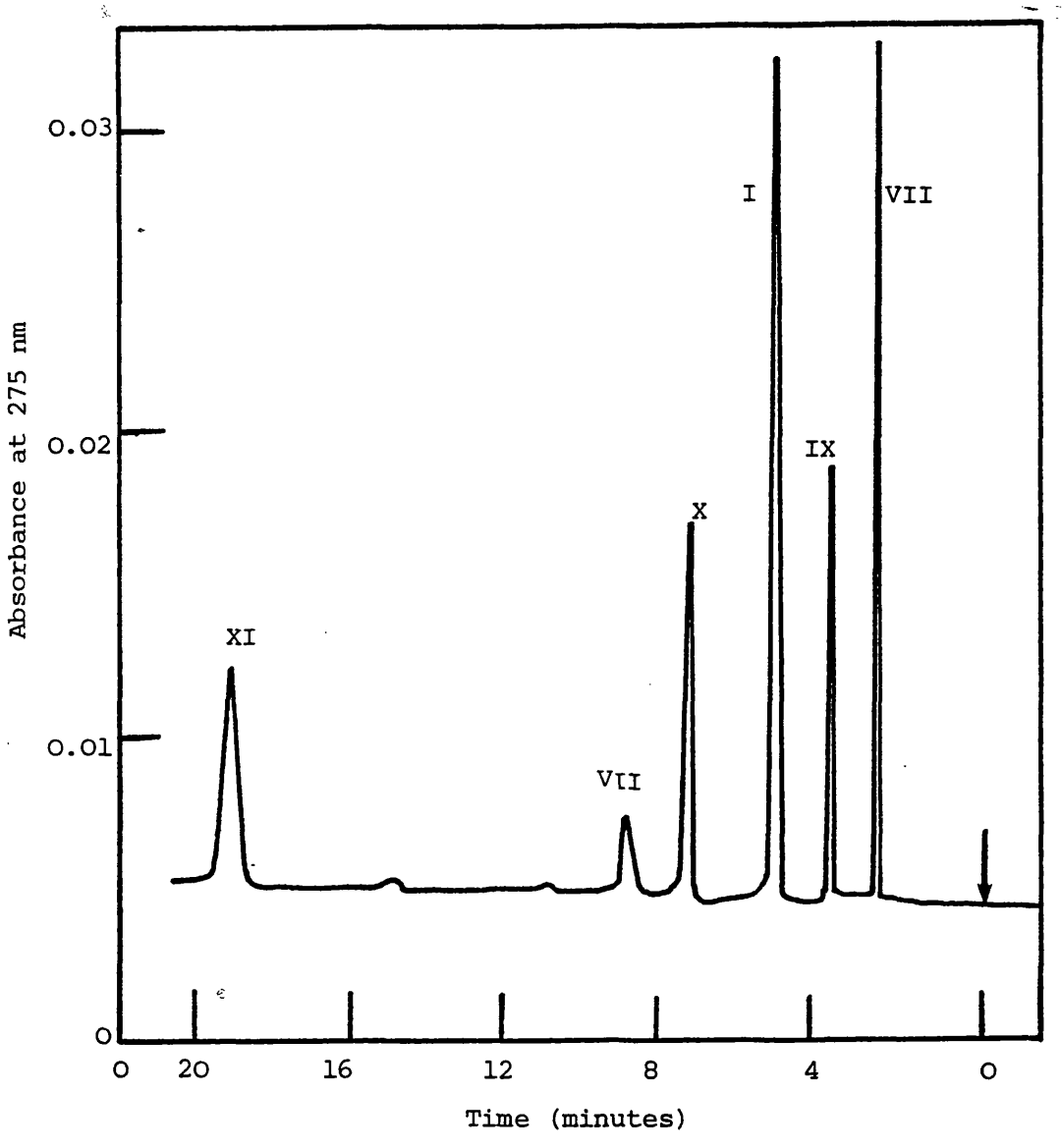


Table 4.28. The concentrations and weights per average tablet of chlorthalidone and atenolol found by assaying eight aliquots of 400 mg taken from ten combined 'Tenoretic' tablets (average weight 413 mg)

Aliquot number	Weight taken (mg)	Chlorthalidone		Atenolol	
		Weight per tablet (mg)	Concentration (%w/w)	Weight per tablet (mg)	Concentration (%w/w)
1	419.5	24.9	6.01	98.1	23.7
2	420.3	24.9	6.01	98.2	23.8
3	403.6	25.1	6.08	96.7	23.4
4	413.2	25.1	6.07	97.6	23.6
5	429.0	25.6	6.18	97.8	23.7
6	413.3	25.4	6.14	97.0	23.5
7	433.2	25.5	6.18	97.6	23.6
8	415.9	24.8	6.01	96.5	23.3
Mean			6.10		23.6
Coefficient of variation (%)			1.13		0.66
Standard error			0.40		0.23

Fig. 4.53. The relationships between the peak areas of atenolol and chlorthalidone and concentrations injected. Conditions as Fig. 4.52.

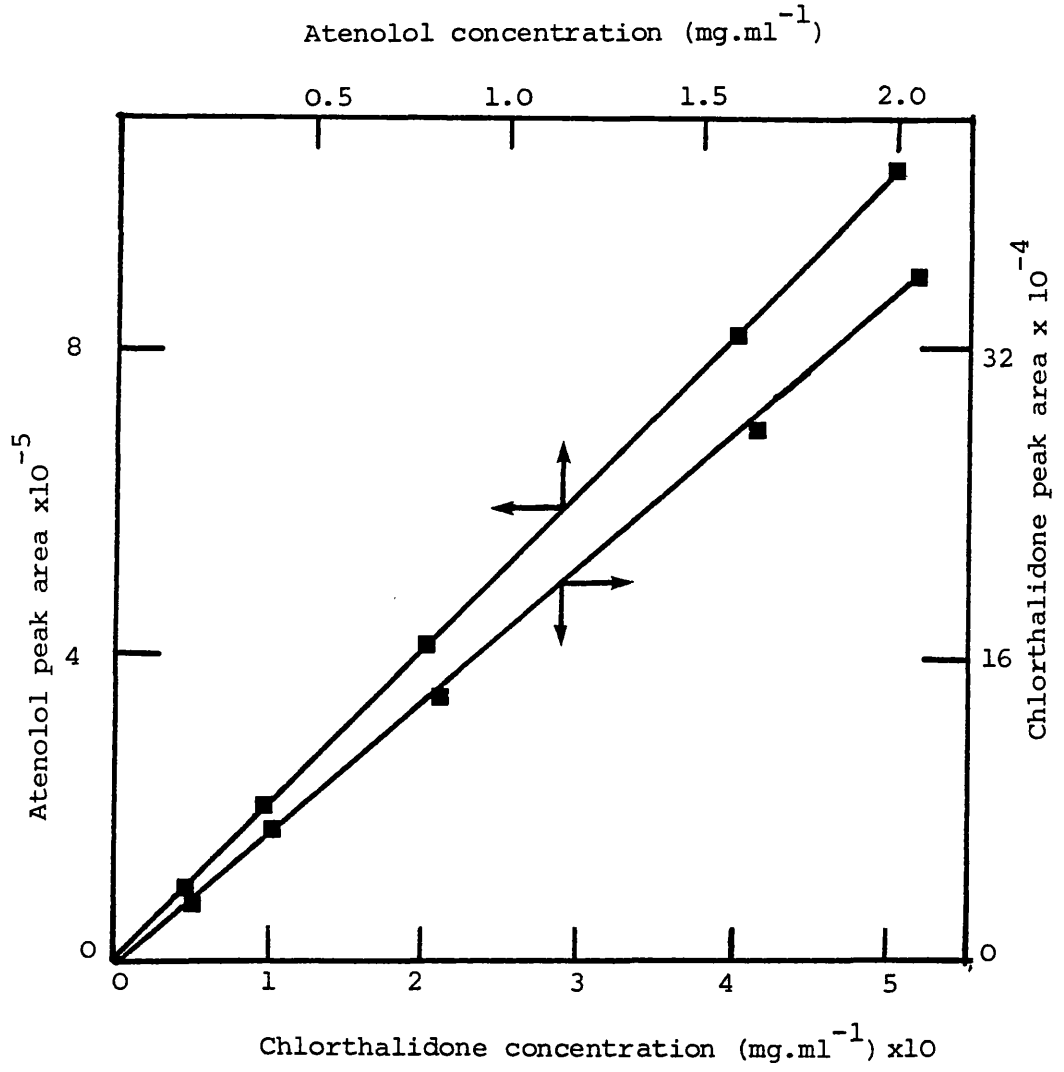
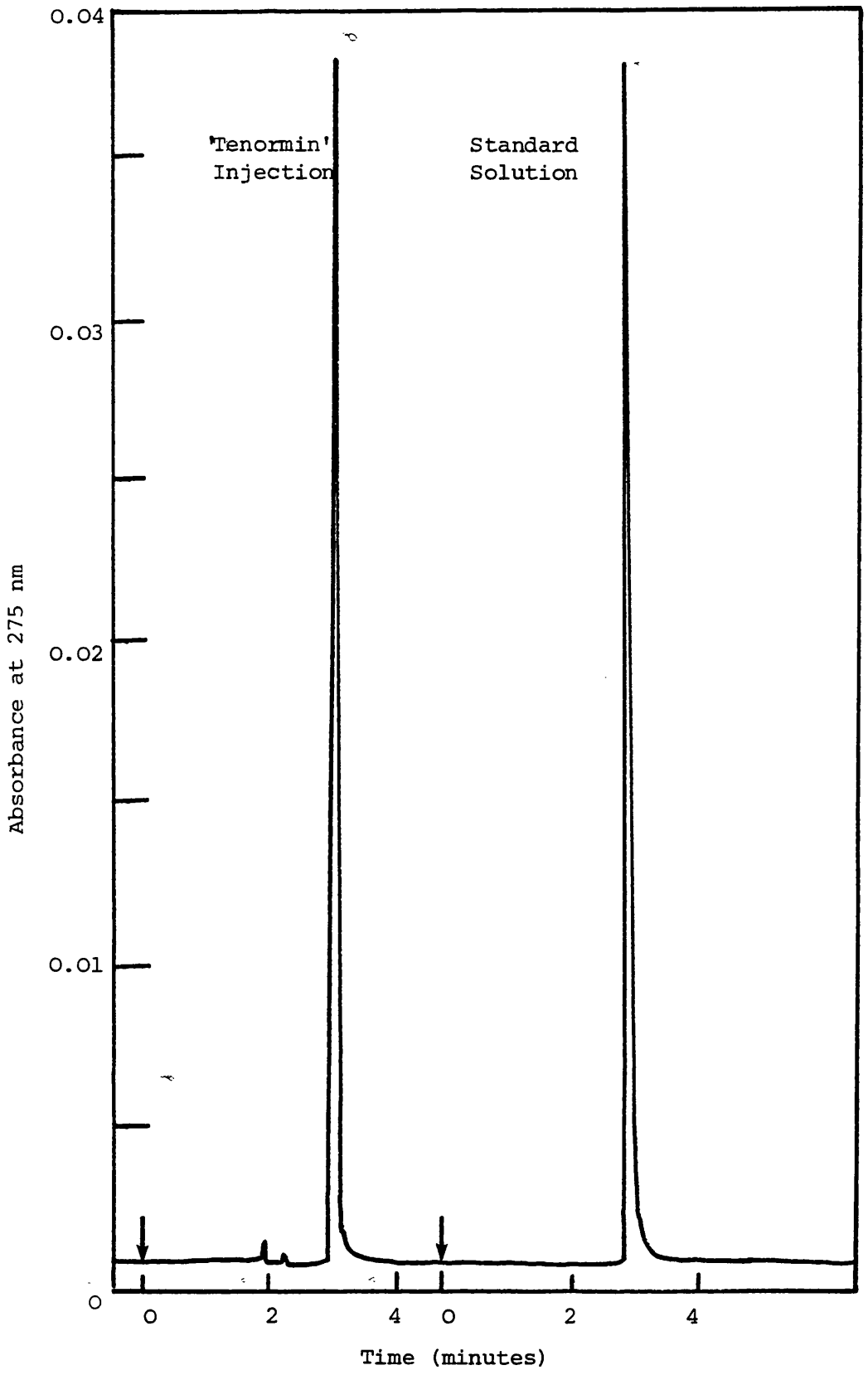


Fig. 4.54. Chromatograms of 'Tenormin' injection (diluted 1 in 10) and a standard solution containing 1 mg.ml^{-1} atenolol. Conditions as Fig. 4.52.



the column when a mobile phase of 25 % v/v acetonitrile and 4 x 10^{-3} $mol.dm^{-3}$ SOS at pH 2.0 was employed. Excellent separation of the four components was achieved by maintaining the SOS concentration at 4 x 10^{-3} $mol.dm^{-3}$ and increasing the acetonitrile concentration to 40% v/v. Although these conditions produced excellent peak shape for three of the components propranolol however eluted as a broad assymmetric band. This poor peak shape was attributed to dissociation of the very hydrophobic propranolol-SOS ion-pair in the hydrophobic stationary phase (136). Generally, (Section 1.4.3), dissociation in the stationary phase is suppressed in reversed phase ion-pair systems by inorganic ions, (in this case SO_4^{2-}), in the mobile phase. Dissociation of propranolol-SOS ion-pair has been suppressed in this study by the introduction of a competing ion, i.e. tetrabutylammonium, which also reduces the retention of propranolol.Tables 4.29 & 3.42 show the effect of tetrabutylammonium phosphate concentration on the retention and plate height of propranolol. Optimal retention of propranolol and acceptable peak shape was achieved with a mobile phase concentration of 10^{-3} $mol.dm^{-3}$ TBAP. It is interesting to note that TBAP had little effect on the retention and peak shape of the aniline derivative, indicating that the 5-trifluoromethyl-2,4-disulphonamiloaniline-SOS has a much lower dissociation constant. (Table 3.42).

During the separation development it was found that bendrofluazide was unstable when dissolved in a mixture of acetonitrile-water (4:6), and significant amounts of 5-trifluoromethyl-2,4-disulphonamideoaniline were detected after storage for one hour at room temperature. To determine a suitable solvent for the

Table 4.29. The effect of tetrabutylammonium phosphate concentration on the measured number of theoretical plates, N, and plate height, h, of the propranolol peak.

Tetrabutylammonium phosphate concentration ($mol.dm^{-3}$)x10^3	N*	H
0.00	338	0.592
0.20	1463	0.135
0.40	2789	0.072
1.00	5875	0.035
2.00	4123	0.049

* mean of two determinations.

preparation of 'Indereticʼ samples, the stability of bendrofluazide was determined in five solvents. 20 mgm of bendrofluazide was dissolved in 10 mls of the following solvent, 1. mobile phase, 2. acetonitrile, 3. acetonitrile-water (4:6), 4. buffer at pH 9.6, and 5. ethanol. The solutions were stored at room temperature, 4°C and 37°C for 24 hours and the stability of bendrofluazide assessed by determining the time profile of 5-trifluoromethyl-2,4-disulphonamidoaniline production (Table 3.52). Bendrofluazide was found to be stable at each temperature for 24 hours when dissolved in ethanol, acetonitrile and mobile phase and highly unstable in alkaline buffer and acetonitrile-water (4:6). The poor peak shape of bendrofluazide in acetonitrile and ethanol precluded their use as solvents. Consequently, the standard solutions and 'Indereticʼ extracts were prepared in mobile phase (40% v/v acetonitrile 4 x 10^{-3} $mol.dm^{-3}$ SOS, 0.1% H_2SO_4). For completeness,

58 mg of propranolol was dissolved in 25 mls of mobile phase and samples stored at $4^{o}C$, $37^{o}C$ and room temperature for 24 hours. 1-naphthol was not detected in any of the samples of propranolol and there was no significant difference in the peak heights of propranolol.

Quantitative measurements were made using the Hewlett-Packard Laboratory computer and the coefficients of variation of peak area for propranolol and bendrofluazide were 0.99% and 1.23% respectively. The response was linear with respect to concentration over the ranges 0.01 to 0.05 $mg.ml^{-1}$ bendrofluazide (r=0.999) and 0.32 to 1.60 $mg.ml^{-1}$ propranolol (r=0.999).

The limit of detection of 1-naphthol in propranolol was found to be 200 ppm and the method was applied to the determination of 1-naphthol in 'Inderal'* injections which contain 100 mg. propranolol. Fig. 4.56 is a chromatogram of 'Inderal' injection (diluted 1 in 10 with distilled water) and a standard reference solution of propranolol containing 2000 ppm 1-naphthol which corresponds to the British Pharmacoepia limit.

The described reversed phase ion-pair HPLSC method allows the rapid determination of propranolol and bendrofluazide and has distinct advantages over the British Pharmacoepial method in respect of the determination of degradation products. Additionally it allows the direct determination of bendrofluazide compared with the indirect determination of the free amine (5-trifluoromethyl-2,4-disulphonamidoaniline).

* 'Inderal' is a registered trade mark of Imperial Chemical Industries Limited.

Fig. 4.55. Separation of propranolol, bendrofluazide and their respective degaradation products. Stationary phase: ODS Hypersil (Column H). Mobile phase: 40% v/v acetonitrile, 0.1% v/v H_2SO_4, 4 x 10^{-3} $mol.dm^{-3}$ SOS. Temperature: ambient, Key as Fig . 2.2.

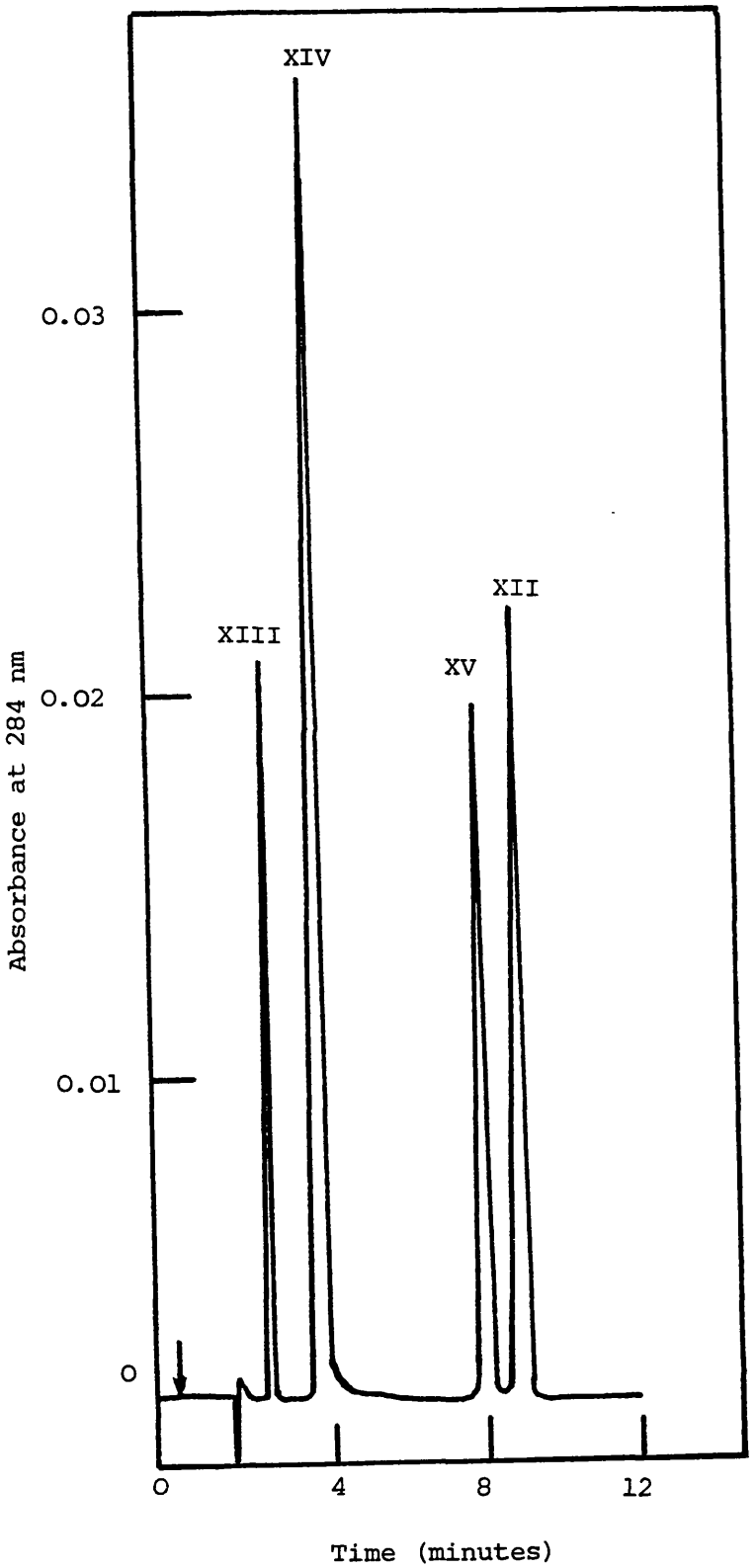


Fig. 4.56. Chromatograms of 'Inderal' injection (diluted 1 in 10) and a standard solution containing 1 mg.ml^{-1} propranolol and 2000 ppm 1-naphthol. Conditions as Fig. 4.55.

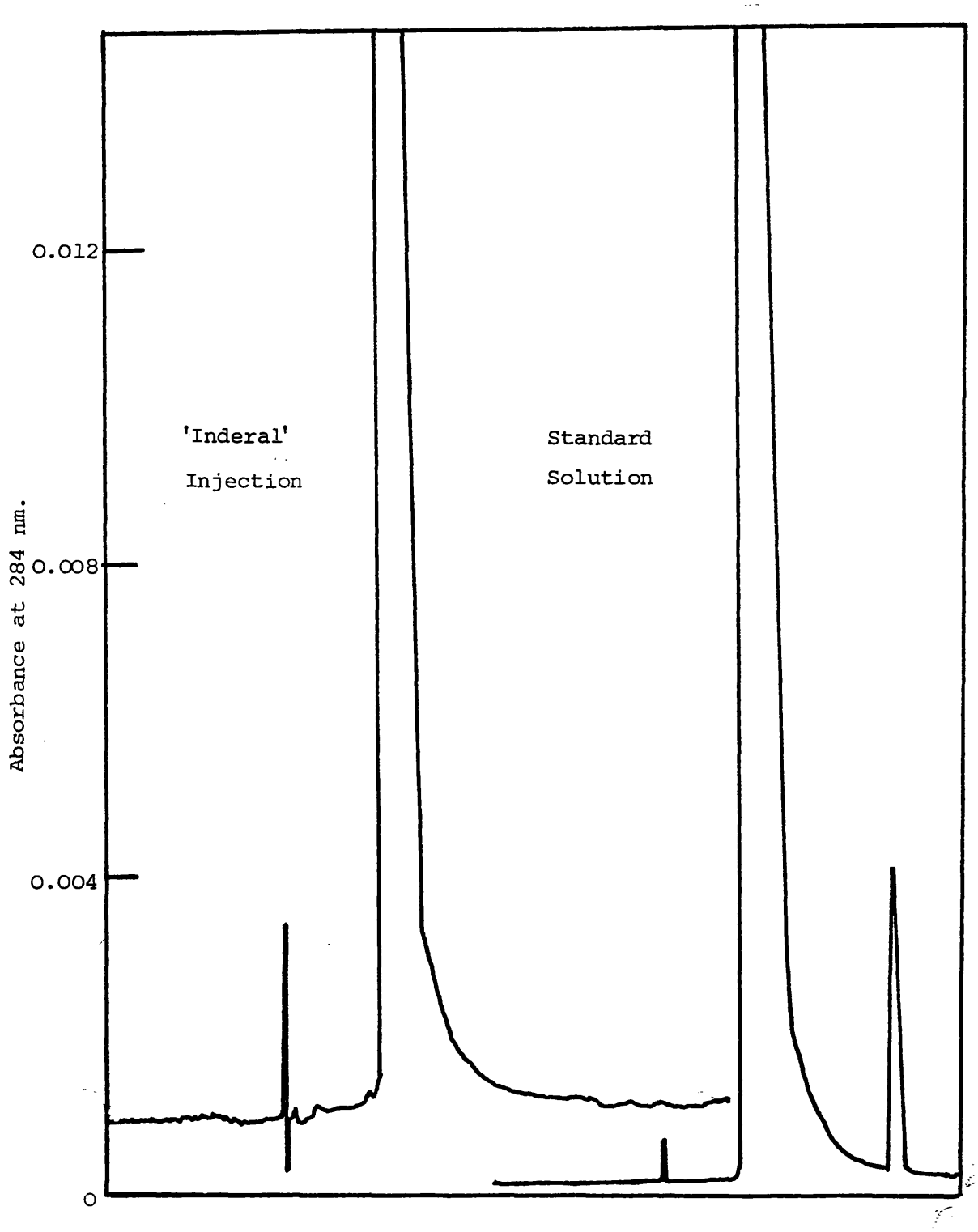
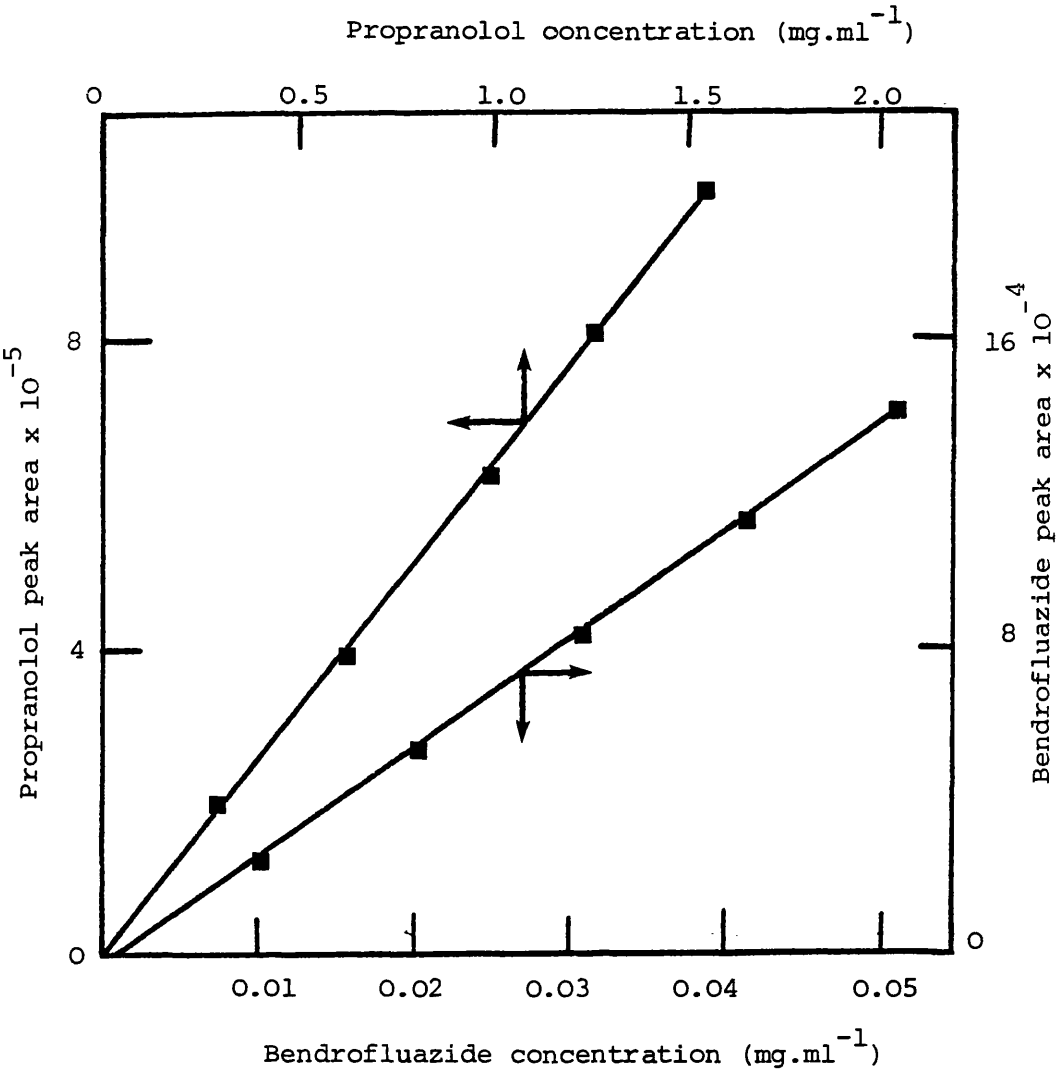


Fig. 4.57. The relationship between the peak areas of propranolol and bendrofluazide and the concentrations injected. Conditions as Fig. 4.55.



4.4.7 Synthetic peptides

Naturally occurring peptides consist of a number of amino acid sub-units joined by amide linkages, and are generally amphoteric due to the presence of both acidic and basic funcational groups. A number of workers (217, 249-255) have shown that reversed phase ion-pair systems are suitable for the separation of peptides. In general acidic mobile phases with anionic pairing-ions are preferred due to the restrictive pH limits (2-7.5) of bonded alkylsilica stationary phases.

Synthetic peptides are prepared from amino-acid sub-units and the correct sequencing is controlled by 'protecting' all but one of the reactive functional groups. Amino groups are protected by such groups as nitro, carbobenzoxy-(CBZ) or tert-butyloxycarbonyl (BOC), phenolic groups are protected by benzyl functions, and carboxyl groups are usually protected by esterification with methyl functions. Consequently 'protected' amino acids and peptides are either acidic or basis rather than amphoteric.

It was required for quality control purposes to separate two tripeptides, nitro-L-arginyl-L-prolyl-azaglycine amide and N-CBZ-O-benzyl-L-tyrosyl-O-t-butyl-D-seryl-L-leucine and a tetrapeptide, Glu.His.Trp.Ser.$NHNH_2$ from their respective synthetic sub-units. It has been argued here and elsewhere (217) specifically for peptides that retention in reversed phase HPLC can be predicted from hydrophobicity parameters. Figure 4.58 shows the separation of the three peptides and their synthetic sub-units with an acidic mobile phase (pH 2.0) containing 70% methanol. It can be

seen that the retention order is related to the hydrophobicity index (Σ f values see Table 4.30 (235)),however the order is modified by both the presence or absence of an ionised amino group, and the nature of any 'protecting' functional group. The tripeptide, N-CBZ-O-benzyl-L-tyrosyl-O-t-butyl-D-seryl-L-leucine in addition to having two hydrophobic sub-units tyrosine ($\Sigma f = 1.70$) and leucine ($\Sigma f = 1.99$), also has three hydrophobic 'protecting' groups - carbobenzoxy, tert-butyl and benzyl, and as a result was not eluted from the column after two hours. The tetrapeptide and the amino-acids containing free ionised amino groups eluted very rapidly close to the solvent front .

The addition of a pairing-ion, SDDS (3×10^{-3} mol.dm^{-3}) enhanced the retention of the bases such that the majority of solutes were separated (Fig. 4.59), however the hydrophobic tripeptide was still uneluted and histdine methyl ester eluted with very poor peak shape. Hence this method was not universally acceptable for the analysis of the solutes and each peptide was subsequently treated individually.

a. Glu-his.trp.ser.NH.NH_2

Potential impurities of the tetrapeptide are the protected amino acids due to incomplete reaction and the amino acid units due to hydrolysis. Thus the ion-pair separation described earlier was modified to be selective for the seven potential impurities (n.b. pyroglutamic acid is unprotected) and the tetra-peptide itself. The peak shape of both histidine methyl ester and histidine itself was poor, due presumably to dissociation effects (Section 1.5.3), and it is recalled from Section 4.3.7 that histidine should form 2:1 complexes with SDDS and will hence have

Table 4.30. Sum of the hydrophobic group contributions of the amino acid side chains, Σf, (ref. 235).

Amino acid	Σf	Amino acid	Σf
Tryptophan	2.31	Lysine	0.52
Phenylalanine	2.24	Glycine	0.00
Leucine	1.99	Aspartic acid	-0.02
Isoleucine	1.99	Glutamic acid	-0.07
Tyrosine	1.70	Histidine	-0.23
Valine	1.46	Threonine	-0.26
Cystine	1.11	Serine	-0.56
Methionine	1.08	Asparagine	-1.05
Proline	1.01	Glutamine	-1.09
Cysteine	0.93		
Alanine	0.53		

Fig. 4.58. Separation of 'protected' peptides and amino acids. Stationary phase: ODS Hypersil. Mobile phase: 0.1% v/v H_2SO_4, 70% v/v methanol. Temperature: ambient. Key: 1, N-BOC-NO_2-arg; 2, NO_2-arg.pro:gly.NH.NH.CO.NH_2; 3, Glu-his-trp.ser.$NH.NH_2$; 4, CBZ-pro; 5, CBZ-trp; 6, CBZ-but-ser; 7, CBZ-benz-tyr; u, unknown. Flow rate: 1.5 ml.min^{-1}. Ambient temperature.

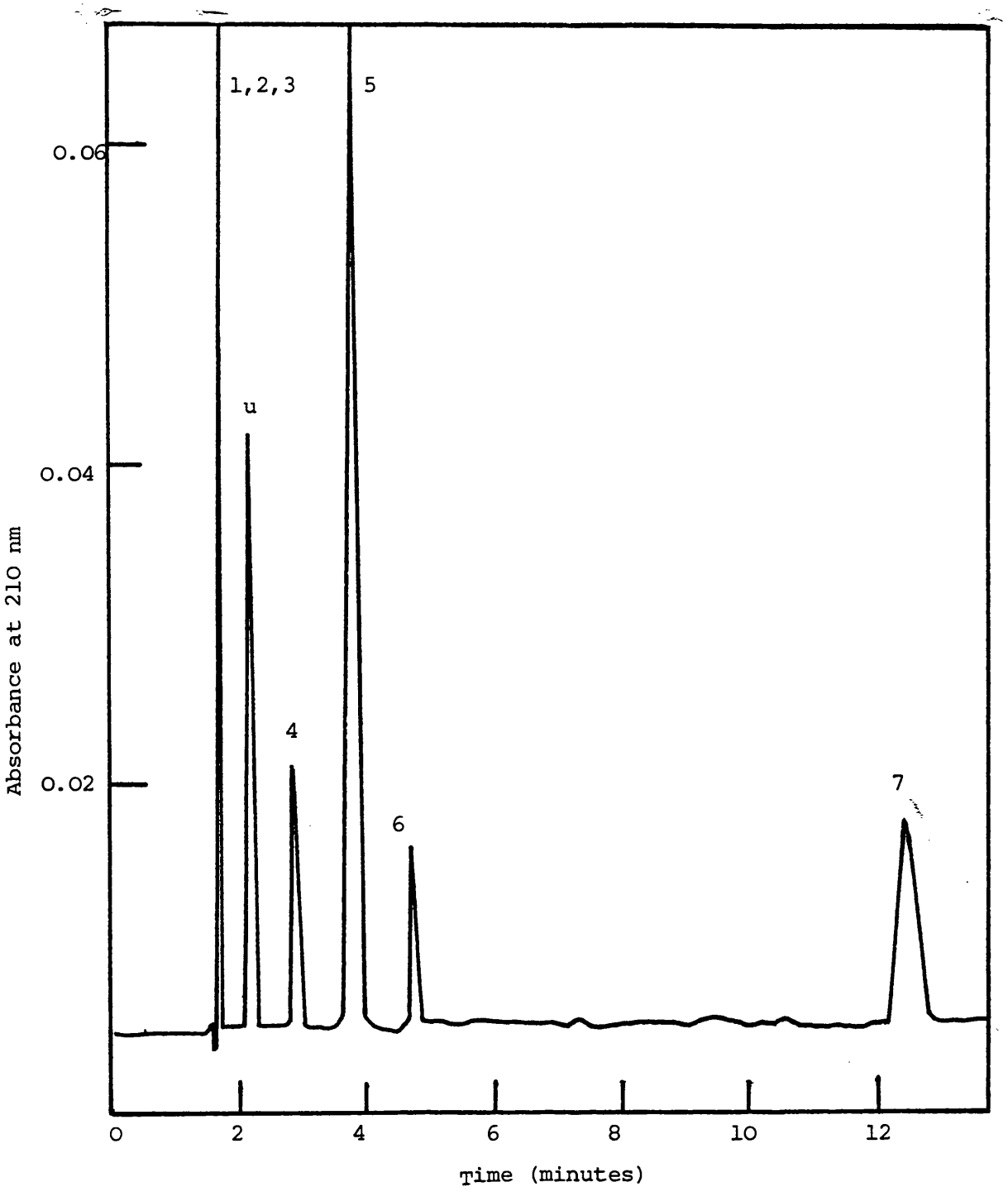
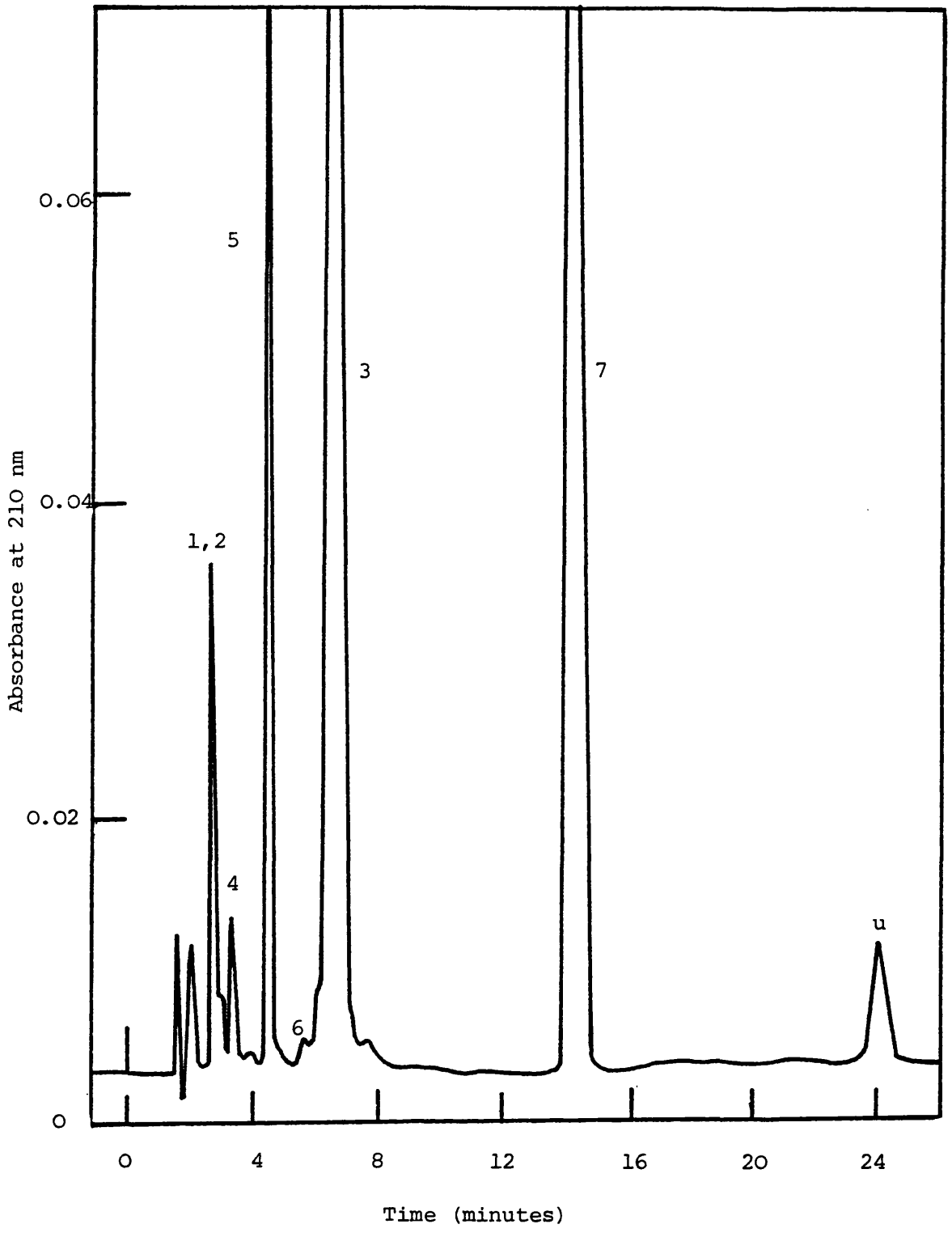


Fig. 4.59. Ion-pair separation of 'protected' peptides and amino acids. Stationary phase: ODS Hypersil: Mobile phase: 0.1% v/v H_2SO_4, 70% v/v methanol, 3 x 10^{-3} mol. dm^{-3} SDDS. Temperature ambient. Flow rate: 1.5 ml.min^{-1}. Key: as Fig. 4.58.



two dissociation constants. Histidine and its methyl ester eluted as a symmetrical band on the introduction of 5 x 10^{-3} $mol.dm^{-3}$ TBAP as a competing species. It can be inferred that the increase in organic modifier used here causes an increase in the dissociation constants of histidine since these effects were not seen when a low organic modifier concentration was used.

Separation of the eight components was achieved by reducing the methanol concentration from 70% v/v to 65% v/v (Fig. 4.60), however it was found that a number of unknown impurities eluting before the tetrapeptide were only partially resolved. To resolve these early eluting impurities, the methanol concentration was further reduced to 55% v/v, and the increase in tetrapeptide retention partially offset by reducing the pairing-ion concentration to 2 x 10^{-3} $mol.dm^{-3}$ SDDS. Fig. 4.61 shows that the seven amino acid sub-units were still well separated under these conditions. The impurity profile of the tetrapeptide was assessed using the two mobile phase systems described using UV detection at 210 nm. and a Hewlett-Packard Laboratory computer. Figures 4.62 and 4.63 show the elution profiles of the tetrapeptides using the chromatographic conditions described in Figs. 4.60 and 4.61, and although traces of histidine, histidine methyl ester, tryptophan, serine and serine methyl ester were detected, the major impurities were apparently side products of the synthesis.

The tetrapeptide was found to be unstable to heating at 90^{o}C for one hour when dissolved in mobile phase and the major degradation product of this treatment eluted after the tetrapeptide (Figs. 4.64 and 4.65). Elution after the main compound indicates the

Fig. 4.60. Separation of the 'protected and unprotected' amino acid sub-units of the tetrapeptide, glu.his.trp.ser.NH.NH_2. Stationary phase: ODS Hypersil. Mobile phase: 65% v/v methanol, 0.1% v/v H_2SO_4 3 x 10^{-3} mol.dm^{-3} SDDS, 5 x 10^{-3} mol.dm^{-3} tetrabutylammonium phosphate. Flow rate: 1.5 ml.min^{-1}. Temperature ambient. Key: as Fig. 4.58 plus 8, glu; 9, ser; 10, Me-ser, 11, his; 12, trp; 13 Me-his.

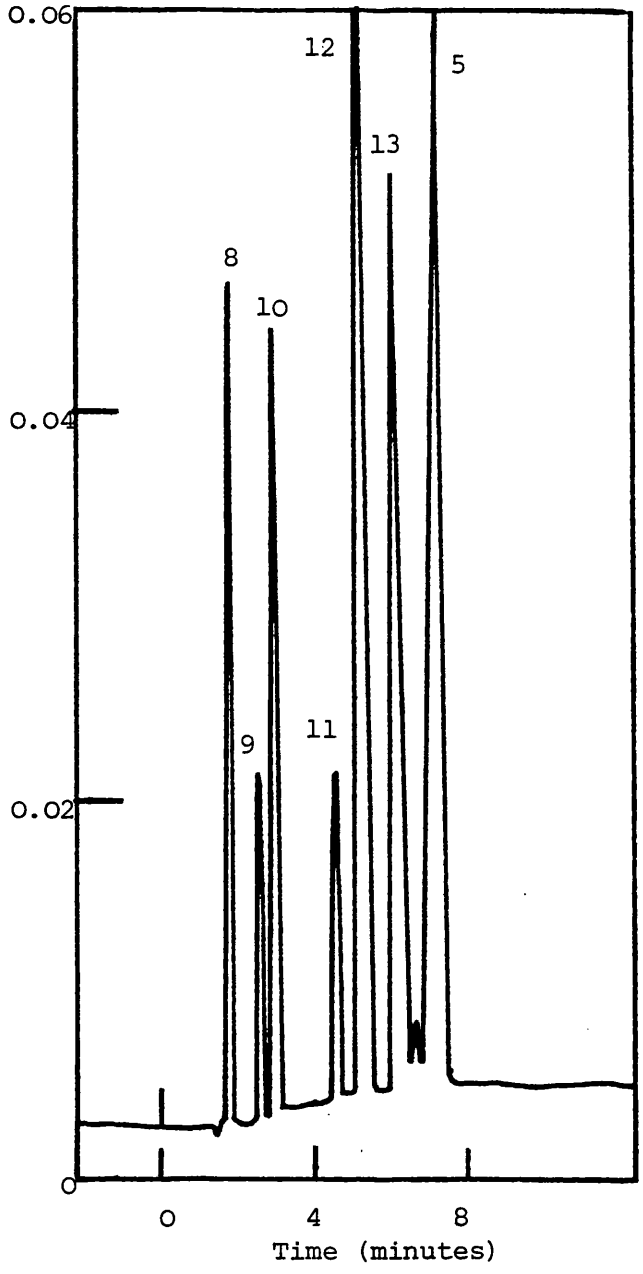


Fig. 4.61. Separation of the 'protected' and'unprotected' amino acid sub-units of the tetrapeptide, glu.his.trp.gly.$NH.NH_2$ Stationary phase: ODS Hypersil. Mobile phase: 55% v/v methanol, 0.1% v/v H_2SO_4, 2 x 10^{-3} $mol.dm^{-3}$ SDDS, 5 x 10^{-3} $mol.dm^{-3}$ tetrabutylammonium phosphate. Flow rate: 1.5 $ml.min^{-1}$. Key as Figs. 4.58 and 4.60.

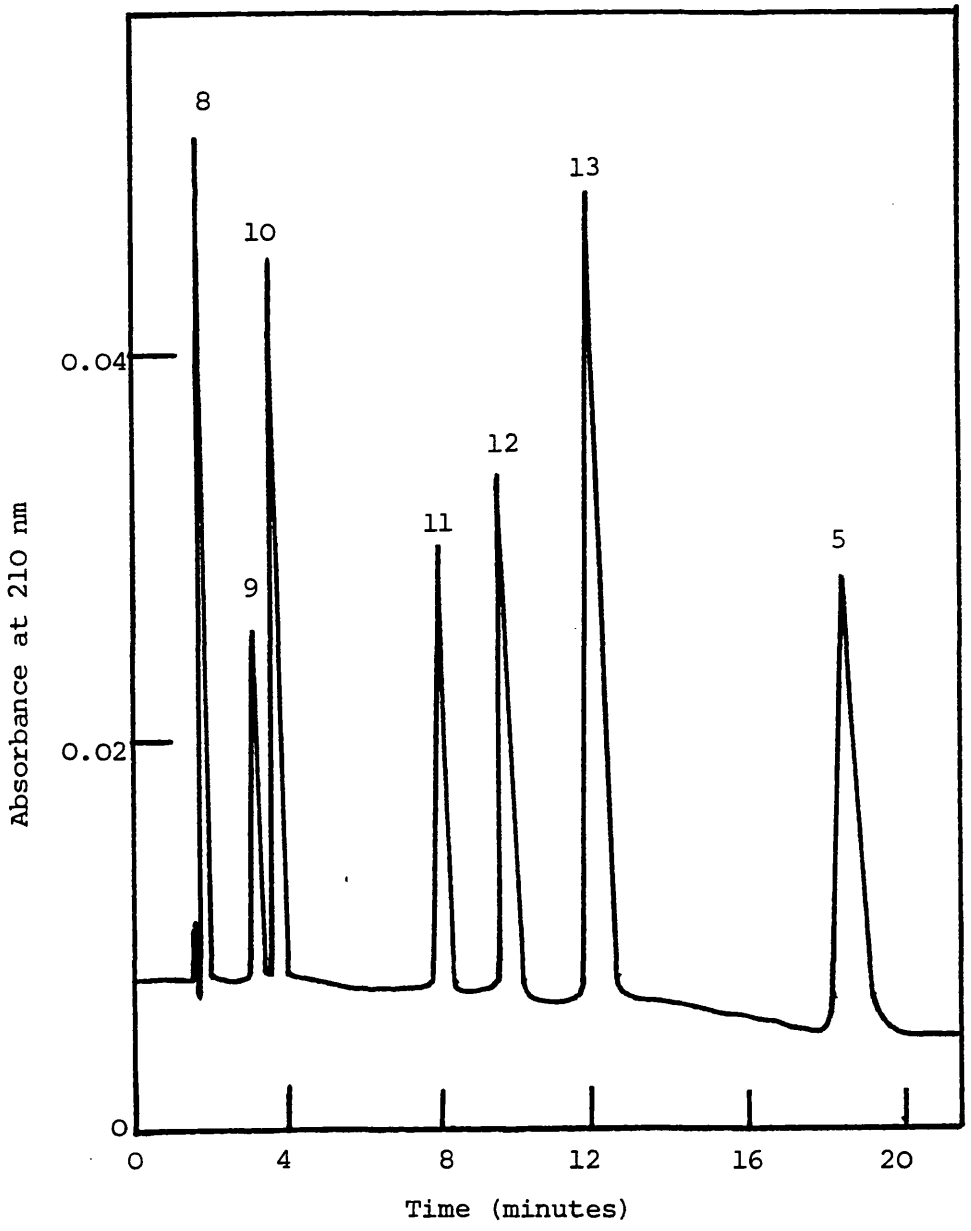


Fig. 4.62. Separation of the impurities in the tetrapeptide, glu.his.trp.ser.NH.NH_2. Conditions as Fig. 4.60. Key: as Fig. 4.58 and 4.60. Solute concentration 5 $mg.ml^{-1}$ freshly prepared and dissolved in mobile phase.

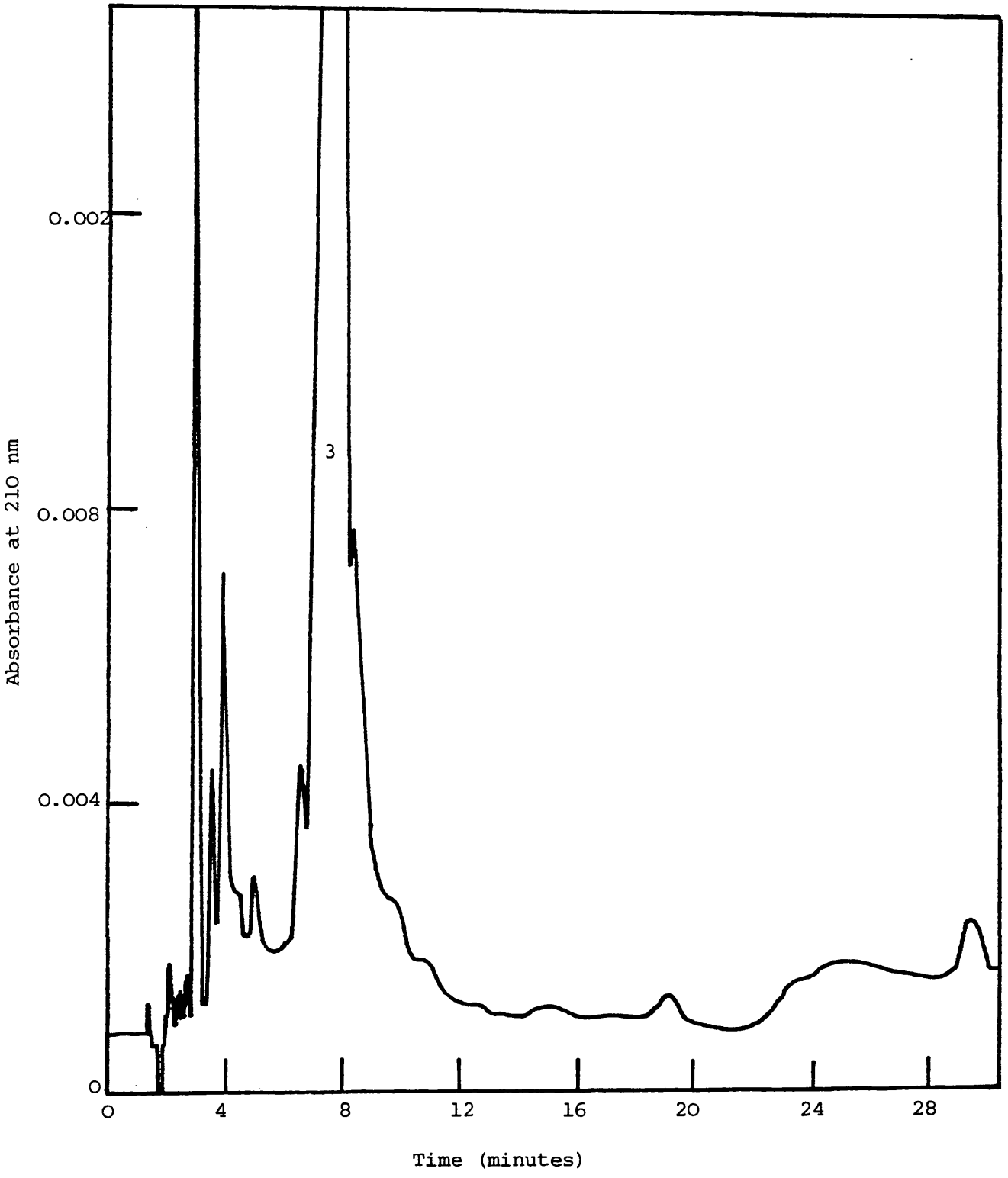


Fig. 4.63. Separation of the impurities in the tetrapeptide, glu.his.trp. ser.NH.NH_2. Solute concentration 5 $mg.ml^{-1}$ freshly prepared and dissolved in mobile phase. Chromatographic conditions as Fig. 4.61. Flow rate: 2.0 $ml.min^{-1}$. Key as Figs. 4.58 and 4.60.

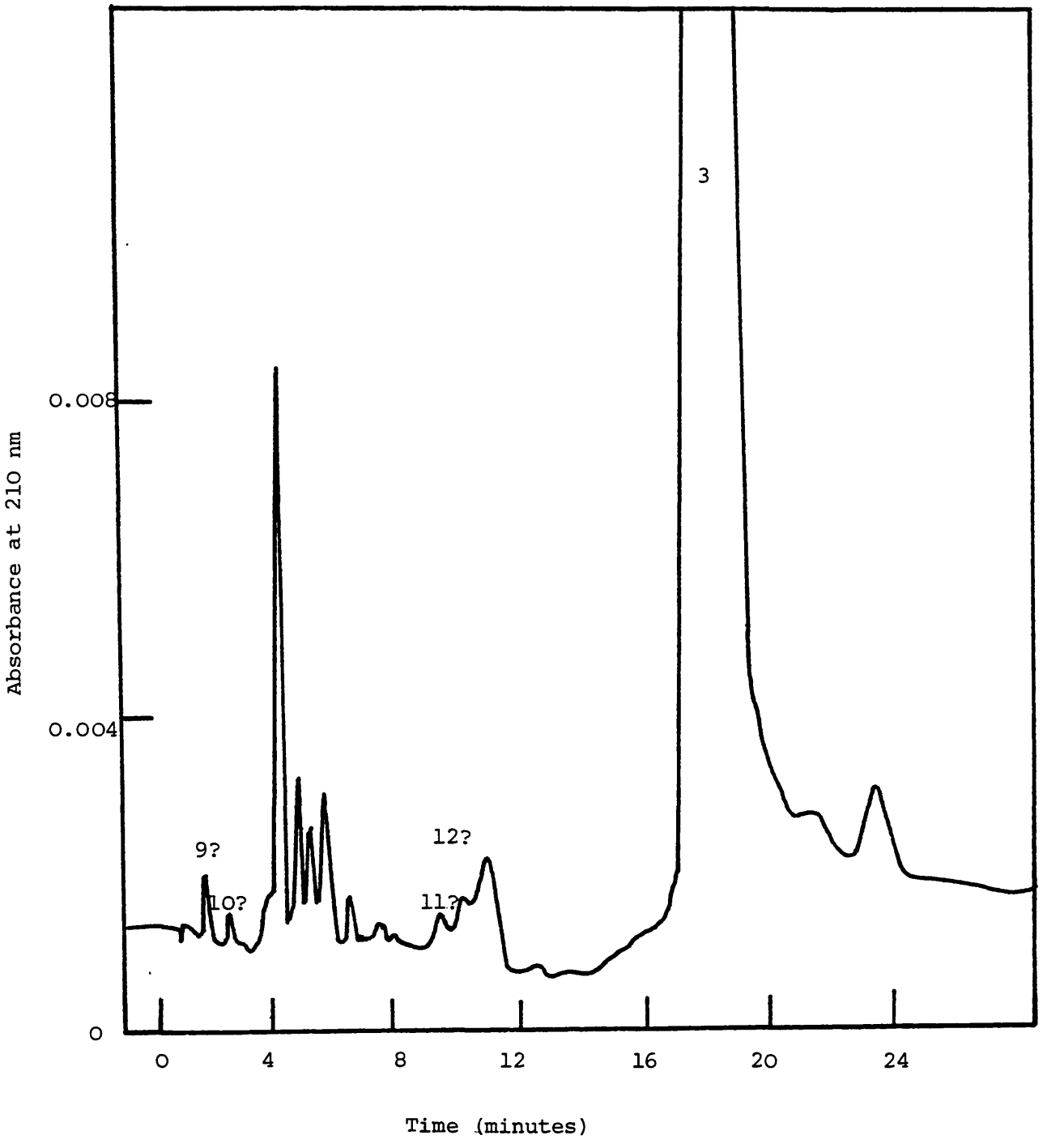


Fig. 4.64. Chromatogram of a degraded sample of the tetrapeptide, glu.his.trp.ser.$NH.NH_2$. Sample 5 $mg.ml^{-1}$ dissolved in mobile phase and heated for 1 hour at 90°C. Conditions as Fig. 4.60. Key as Figs. 4.58 and 4.60.

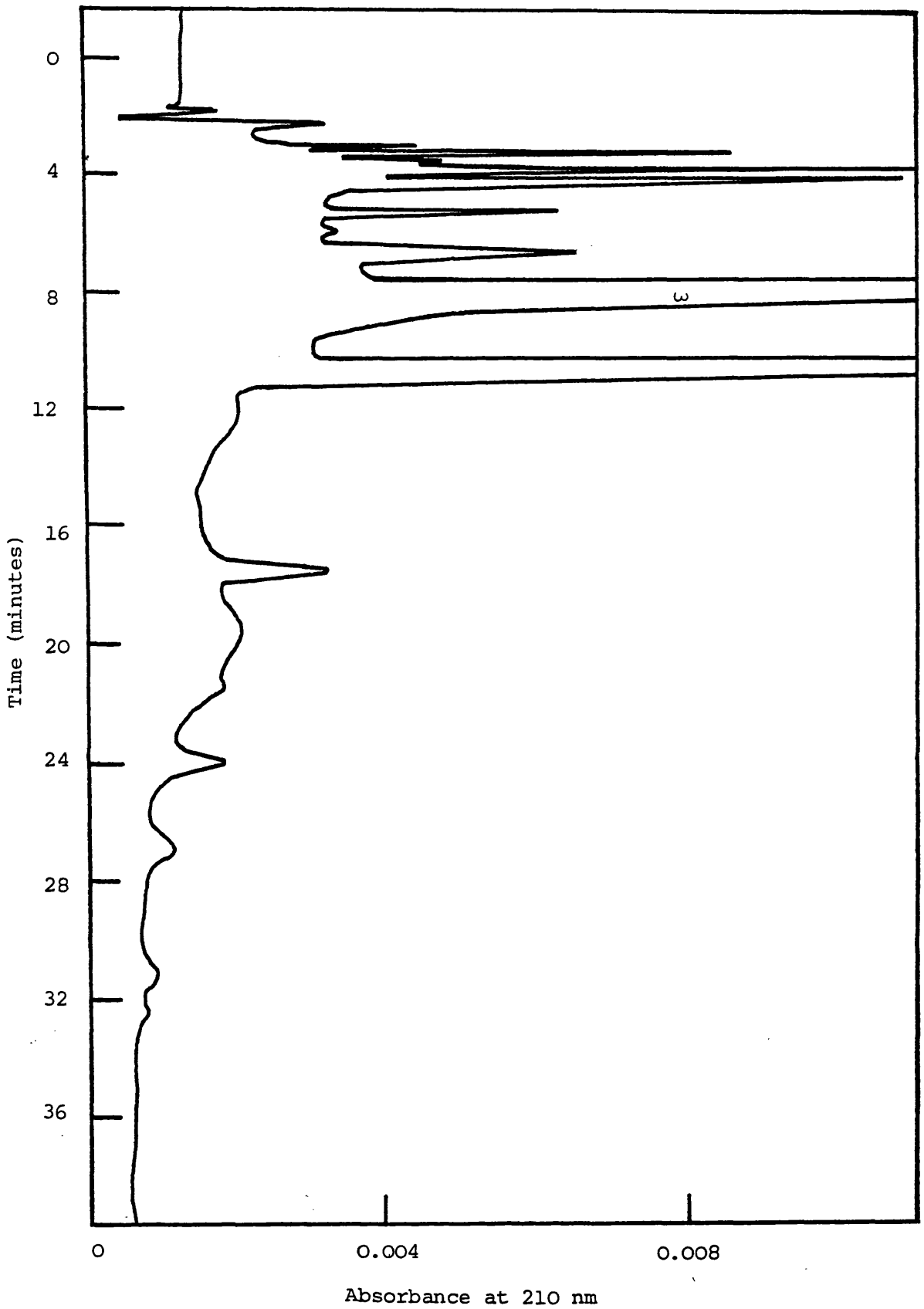
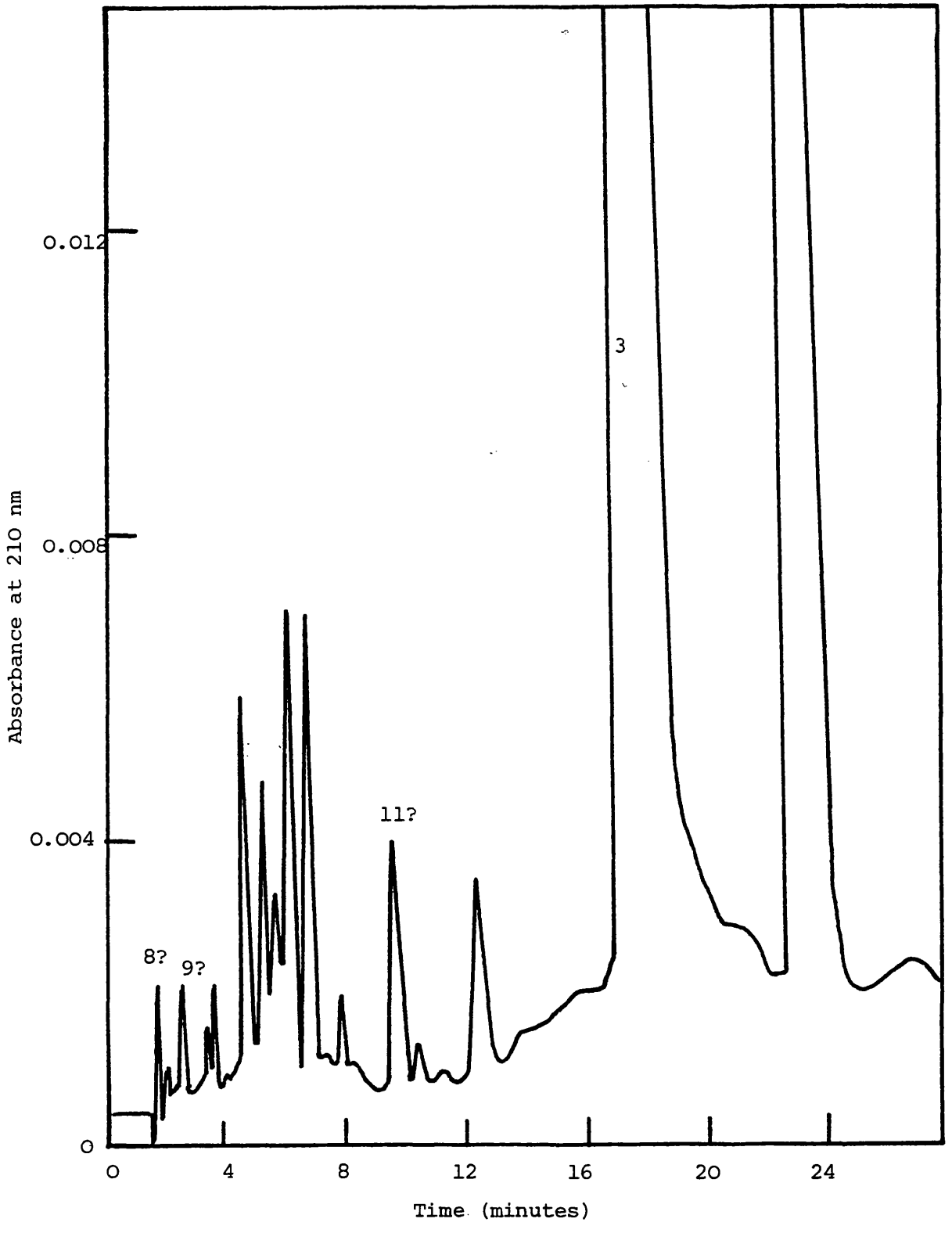


Fig. 4.65. Chromatogram of a degraded sample of the tetrapeptide, glu.his.trp.ser.NH.NH_2. Sample 5 $mg.ml^{-1}$ dissolved in mobile phase and heated for 1 hour at 90°C. Conditions as Fig. 4.61. Key as Figs. 4.58 and 4.60.



loss of a polar portion of the molecule and is probably due to hydrolysis of the terminal pyroglutamic acid moiety which is suggested by an increase in the peak height of a component eluting with the same retention time as pyroglutamic acid. Figure 4.65 shows also the presence of several minor degradation products eluting after the main band, corresponding to the loss of further polar sub-units histidine (Σf =-0.23) and serine (Σf =-0.56), which was confirmed by an increase in the peak heights of compounds eluting with the same retention as histidine and serine.

b. N-CBZ-O-benzyl-L-tyrosyl-O-t-butyl-D-seryl-L-leucine

It can be predicted from Sections 4.3.3 and 4.3.5 that this very hydrophobic peptide would show a dramatic dependence on temperature and organic modifier concentration. Experimentally it was shown that this peptide was uneluted at ambient temperature with a mobile phase containing 70% v/v methanol, however a capacity ratio of 4.58 was obtained if the temperature and organic modifier concentration were raised to 30°C and 75% methanol respectively. With a pairing-ion concentration of 3×10^{-3} $mol.dm^{-3}$ SDDS at pH 2.0 leucine, leucine methyl ester and serine eluted at the solvent front and due to their low UV absorbance were poorly detected. However the aromatic, hydrophobic amino acids and analogues were well separated and easily detected (Fig. 4.66) The majority of the major impurities eluted before the main peak and several of these corresponded to unreacted protected amino acid sub-units, although several major impurities were unidentified (Fig. 4.67). Unlike the other two peptides examined this tripeptide was stable to heating for 1 hour at 90°C dissolved in mobile phase.

Fig. 4.66. Separation of the 'protected' and 'unprotected' amino acid sub-units of the tripeptide, CBZ-benz-tyr.but-ser. leu. Stationary phase: ODS Hypersil. Mobile phase: 75% v/v methanol, 0.1% v/v H_2SO_4 , 3 x 10^{-3} $mol.dm^{-3}$ SDDS. Temperature: 30°C. Flow rate: 2 $ml.min^{-1}$.
Key as Fig. 4.58 plus. 14, leu; 15, Me-leu; 16, tyr.

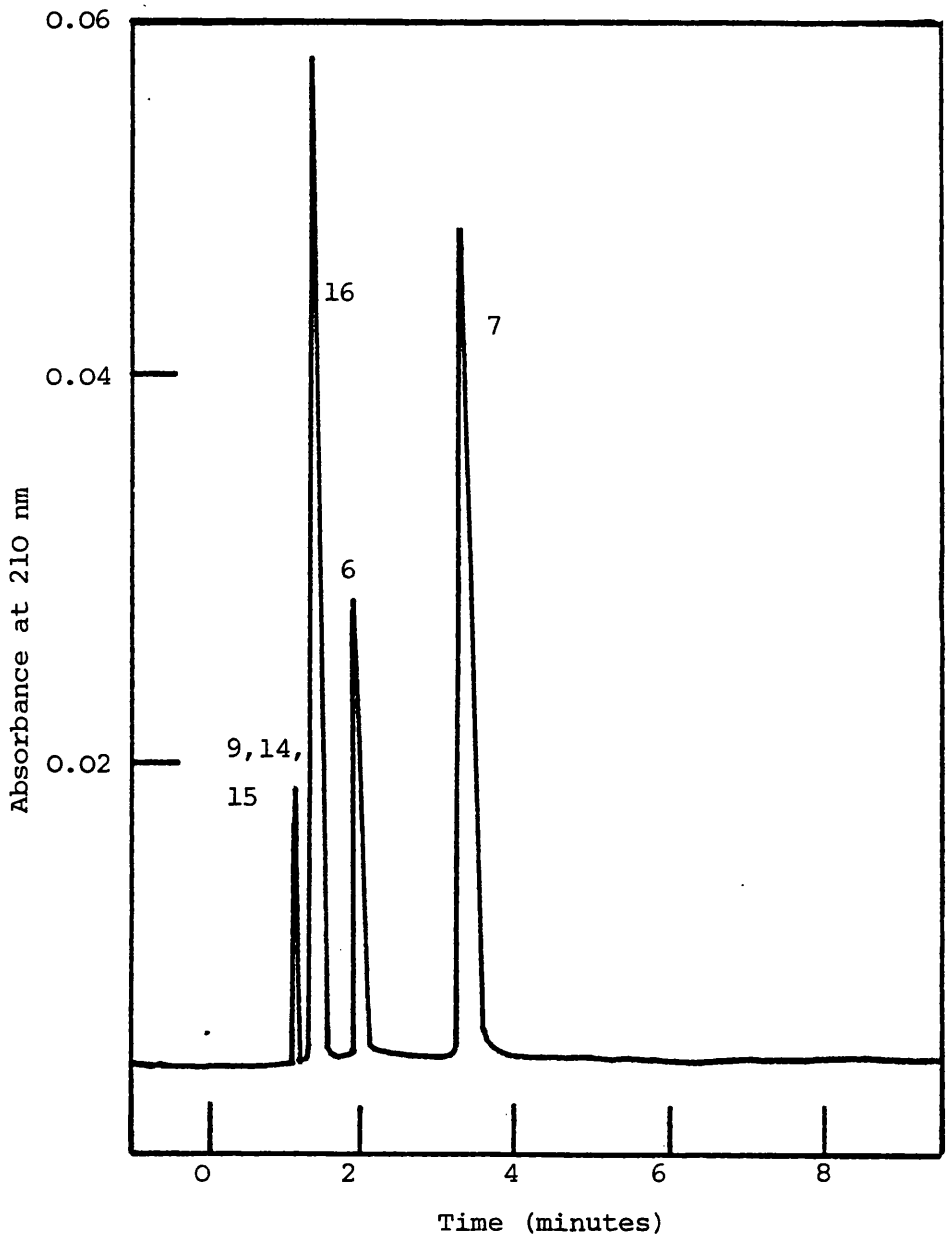
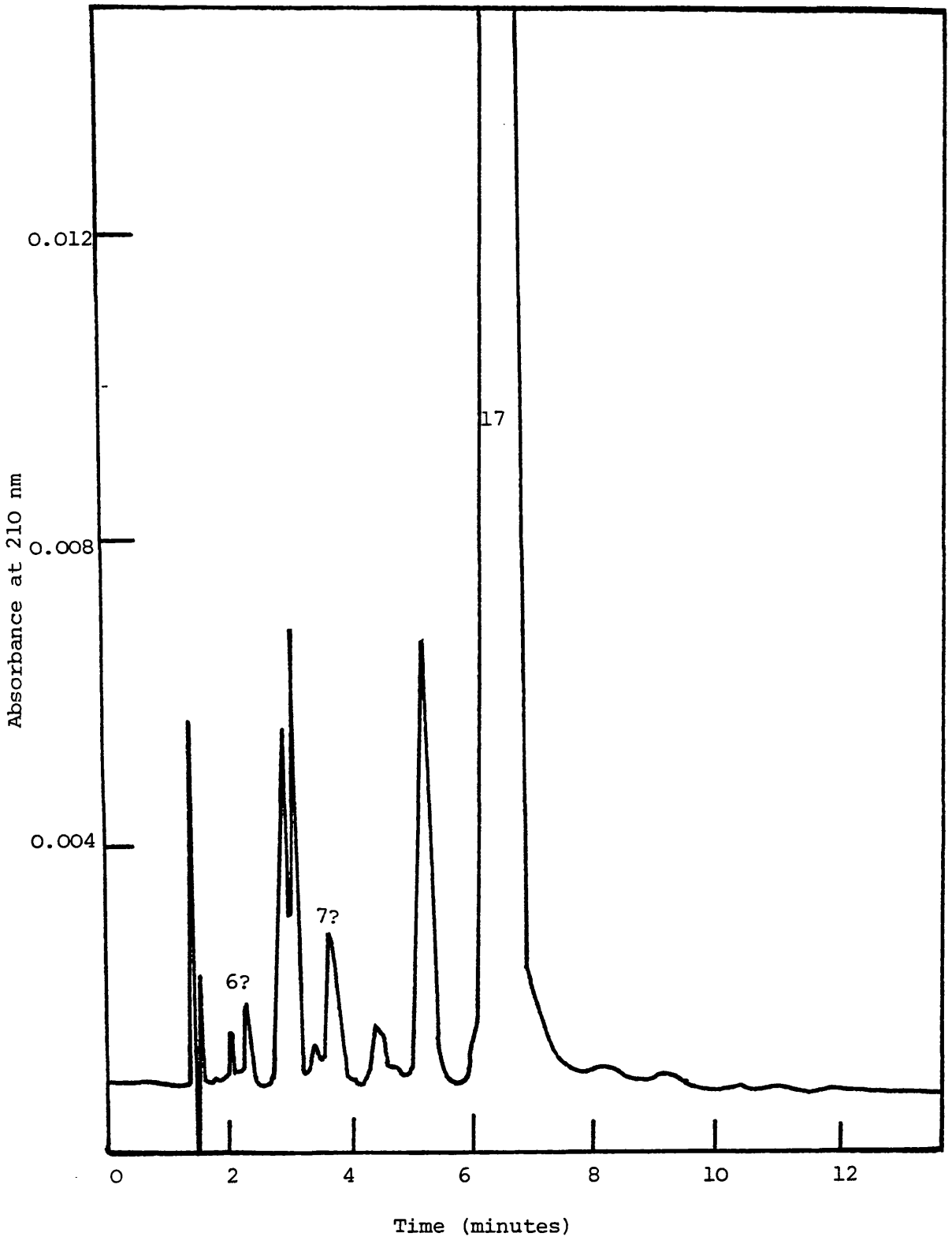


Fig. 4.67. Separation of the impurities in the tripeptide (17,) CBZ-benz-tyr-but-ser.leu. Conditions as Fig. 4.66. Key as Figs. 4.58 and 4.66. Solute concentration 5 $mg.ml^{-1}$ freshly prepared in mobile phase.



c. Nitro-L-arginyl-L-prolyl-azaglycine amide.

This peptide and its sub-units are much less hydrophobic than those described above and these required a much lower methanol concentration to be resolved adequately with a mobile phase containing 47.5% v/v methanol, 2 x 10^{-3} mol.dm^{-3} SDDS at pH 2.0 arginine eluted at the solvent front, however the other components were well separated (Fig. 4.68).

Significant amounts of CBZ-proline were detected in the sample of this tripeptide, however the major impurity was attributed to a peak eluting with a much larger retention than the peptide itself. This impurity is much more hydrophobic than any of the combinations of the sub-units in the parent compound and is assumed to be either a polymer of the parent peptide or totally unrelated (Fig. 4.69).

Both the major impurity and the tripeptide itself were found to be unstable to heat for 1 hour at 90°C and resulted in an increase in the area of the peak with a capacity ratio of 0.47 and the appearance of second peak with a capacity ratio of 1.36 (Fig. 4.70). The most likely route of degradation is hydrolysis to yield nitroarginine and proline azaglycine amide. This hypothesis could not be confirmed due to the lack of authentic sample however it is supported by the application of a group contribution approach. The log κ value for arginine itself is -0.50 whereas the log κ value for the first degradation peak is -0.33 which is consistent with a τ value of 0.17 for nitro (see Table 3.1). The differences in the peak areas of the two degradation products is due to their different UV absorbances.

Fig. 4.68. Separation of the tripeptide NO_2-arg.pro-gly.NH.NH.$CONH_2$ and its 'protected' and 'unprotected' amino acid sub-units. Stationary phase: ODS Hypersil. Mobile Phase: 47.5% v/v methanol, 0.1% v/v H_2SO_4, 2 x 10^{-3} $mol.dm^{-3}$ SDDS. Temperature: 30°C. Flow rate: 2.0 $ml.min^{-1}$. Key as Fig. 4.58 plus 18, pro; and 19, arg.

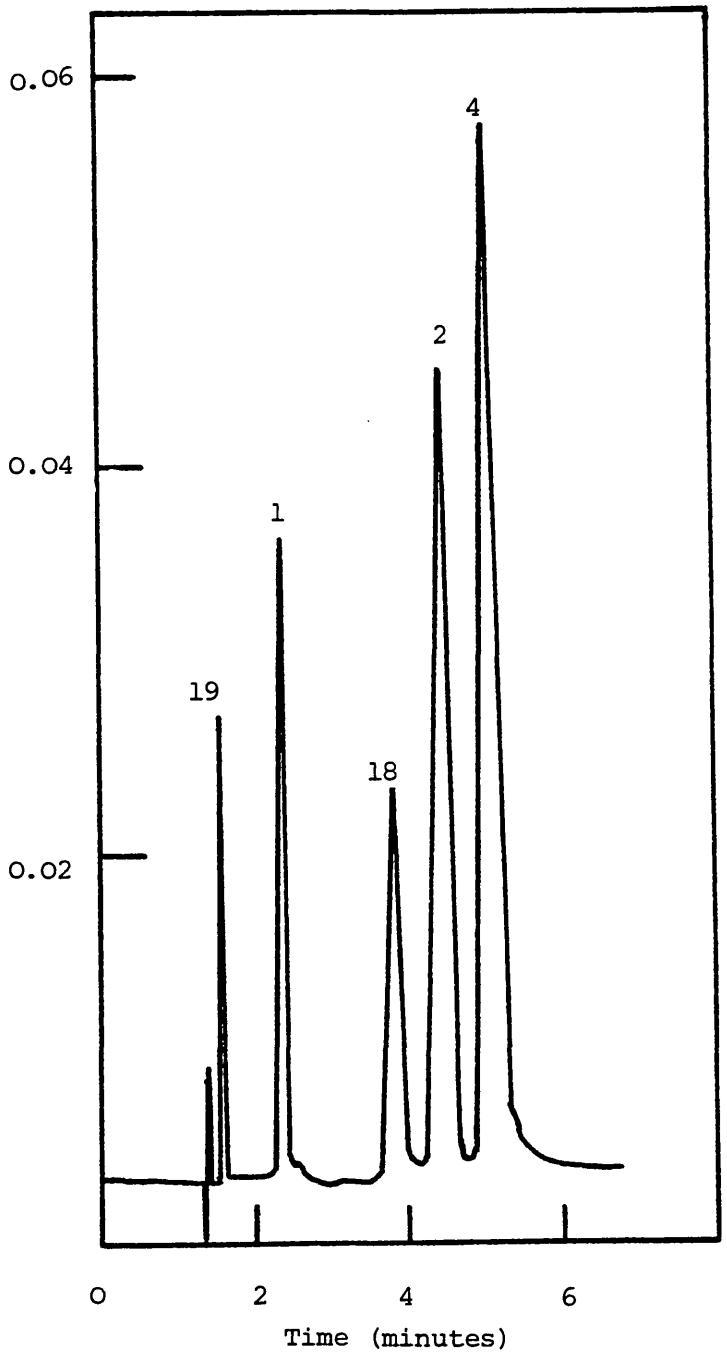


Fig. 4.69. Separation of the impurities of the tripeptide, NO_2-arg.pro.gly.NH.NH.$CONH_2$. Solute concentration 5 $mg.ml^{-1}$ freshly prepared in mobile phase. Conditions as Fig. 4.68. Key as Figs. 4.58 and 4.68.

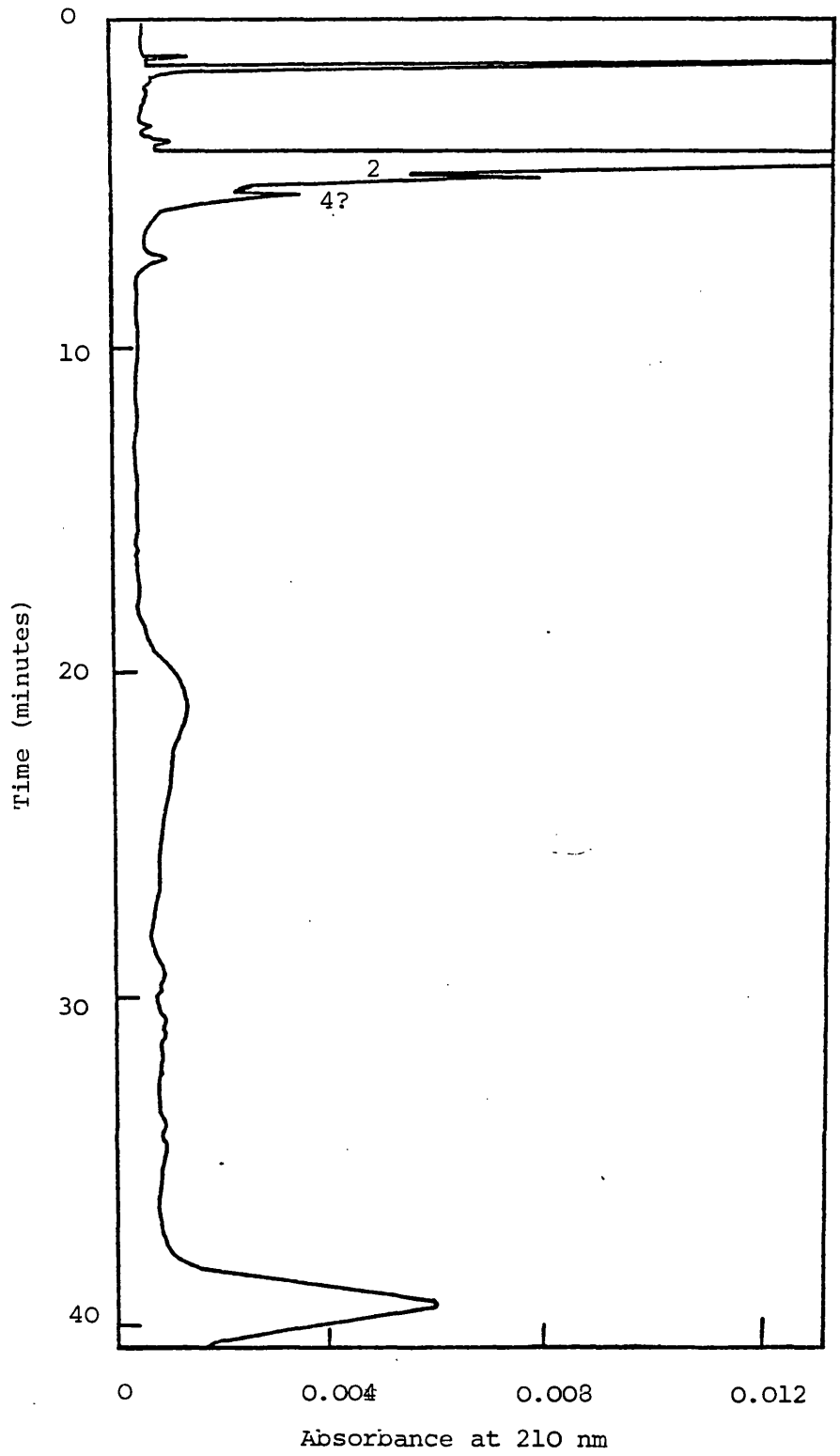
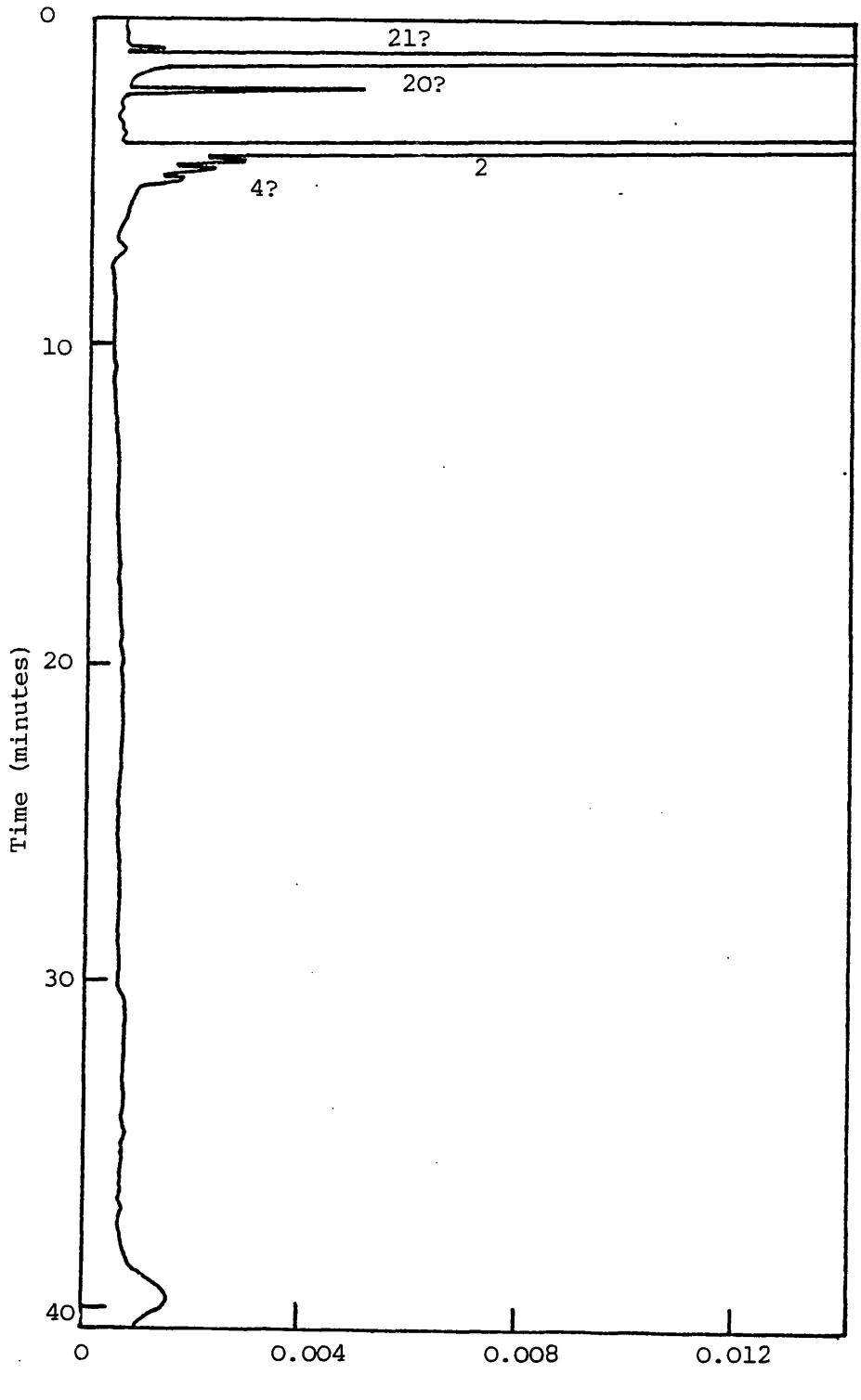


Fig. 4.70. Chromatogram of a degraded sample of the tripeptide, NO_2-arg.pro.gly.NH.NH.$CONH_2$. Sample: 5 $mg.ml^{-1}$ in mobile phase and heated for 1 hour at 90°C. Conditions as Fig. 4.68. Key: as Figs. 4.58 and 4.68 plus: 20, proline azaglycineamide; 21, nitroarginine.



4.5 Optically Active Pairing-Ions

Column liquid chromatography has been applied extensively to the resolution of enantiomeric pairs and the methods have included the use of diasteriomeric derivatisation (203, 256, 257), optically active ion-exchangers (258), charge transfer complexes(259), ligand exchange (260) and metal chelate surfactants (261, 262). Despite the fact that diasteriomeric crystallisation involving ion-pair formation is widely used for the bulk resolution of enantiomers, no reports exist on the use of optically-active pairing-ions in HPLC for the chromatographic resolution of D and L isomers (203). The crystallisation technique involves the addition of an optically active acid or base to a racemic mixture resulting in the preferential crystallisation of one diasteriomeric salt (ion-pair) (263). Camphorsulphonic acid is widely used as a resolving agent in diasteriomeric crystallisation and its potential as a chromatographic resolving agent in reversed phase ion-pair HPLC has been assessed in this present study.

4.5.1 D-camphorsulphonic acid.'

D-camphorsulphonic acid is a strong acid ionised at virtually all pHs and is capable of ion-pair formation with cations at low pH. The effect of D-camphorsulphonic acid on the retention of a number of enantiomeric amino-acids in a mobile phase of methanol: water(1:9) at pH 2.0 on a reversed phase column has been investigated. It can be seen from Table 4.31 that D-camphorsulphonic acid acts as a pairing-ion, and increases the retention of amino-acids; however it does not behave differently from an alkyl sulphate pairing-ion with the exception of histidine which eluted before DOPA (cf. Section 4.3.7) indicating

only 1:1 interaction here. With the exception of the very hydrophobic tryptophan no difference in the capacity ratios of the D and L isomers was observed. The capacity ratios of D-tryptophan and L-tryptophan were 49.9 and 49.6 respectively with a mobile phase concentration of 10^{-2} mol.dm^{-3} D-camphorsulphonic acid. This corresponded to a selectivity coefficient of 1.05 and well over one million theoretical plates would be required to separate the two isomers of tryptophan (Eq. 11). Higher concentrations of D-camphorsulphonic acid were not possible due to the high UV background.

The inability of D-camphorsulphonic acid to resolve enantiomers in reversed phase-ion-pair HPLC are probably due to the different thermodynamic processes involved compared with crystallisation techniques which utilise different crystal lattice energies, and suggests that resolution by ion-pair formation in the mobile phase requires selectivity arising from different ion-pair formation constants, rather than different ion-pair distribution constants (262). We have shown previously (Section 3.3.1) that ion-pair formation constants can be influenced by steric factors and suggests that selective ion-pair formation between optically active pairing-ions and racemic solutes will occur when their respective assymmetric centres are both close to the charge centres. This condition is not satisfied for D-camphorsulphonate-amino-acid pairs. Clearly this selective ion-pair formation will be enhanced, if the charge centre on the pairing-ion is sterically hindered by substitution close to the charge centre.

Table 4.31. The capacity ratios of amino-acids at different mobile phase concentration of D-camphorsulphonic acid. Stationary phase: Spherisorb S5 ODS (column J). Mobile phase: Methanol:water (1:9), pH 2.0 (0.1% H_2SO_4). 30°C.

Solute	D-camphorsulphonic acid concentration ($mol.dm^{-3} \times 10^3$)		
	1.00	3.00	10.0
L-Trp	6.40	26.5	49.9
D-Trp	6.40	26.5	49.6
L-5HTP	2.37	10.5	18.4
D-5HTP	2.37	10.5	18.4
L-DOPA	0.84	3.20	6.40
D-DOPA	0.84	3.20	6.40
L-His	0.12	2.05	4.00
D-His	0.12	2.05	4.00

4.5.2 D,L-carboethoxyheptyltrimethylammonium methylsulphonate.

From the above arguments it would appear that an appropriate pairing-ion for the resolution of optical isomers would be aprotic with a sterically hindered charge centre which is close to the optical centre. In an attempt to satisfy these requirements, D,L-carboethoxyheptyltrimethylammonium was synthesised in which the charged ammonium group is close to the assymmetric centre and is shielded by a carboethoxy group. This compound was synthesised in three stages from D,L-2-aminooctanoic acid and the overall yield was *ca*. 30%, however attempts to resolve either the final product or its intermediate enantiomers proved unsuccessful.

A preliminary investigation of the potential of this pairing-ion was made by investigating the retention behaviour of a number of amino-acids in a reversed phase system. Previous work (Section 4.3.7) has shown that high pHs are required if amino proprionic acids are to be retained by ion-pair formation with quaternary ammonium salts, hence a mobile phase of pH 9 was employed and the hydrolysis of the stationary phase prevented by the use of a pre-column.

D,L-carboethoxyheptyltrimethylammonium enhanced the retention of a number of amino-acids studied when added to a mobile phase of 0.13 $mol.dm^{-3}$ NH_4Ac adjusted to pH 9.0 with NH_3 at a concentration of $5x10^{-3} mol.dm^{-3}$. The enhancement of retention was much lower than would be expected for pairing-ions of this type, and suggests that carboethoxy shields the charge centre, (as predicted), resulting in lower ion-pair formation constants, which satisfies one of the criteria discussed above. The enhancement of 3-hydroxy-

kynurenine is seen to be greater than for the other solutes investigated and this is taken to be indicative of selective ion-pair formation (Figs. 4.71 and 4.72). Further evidence of selective ion-pair formation is seen in Fig. 4.73 which shows the poor peak shape of DOPA after the addition of D,L-carboethoxyheptyltrimethylammonium to the mobile phase (0.13 mol.dm^{-3} NH_4COO).

Clearly optically active carboethoxyhetptyltrimethylammonium has potential as a pairing-ion for the resolution of enantiomeric pairs by reversed phase HPLC, however further work is needed to improve its synthetic yield which is low (<30%) due to hydrolysis of the ester linkage and to resolve the racemic mixture of the pairing-ion.

Fig. 4.71. Separation of four amino acids. Stationary phase: ODS Hypersil. Mobile phase: 0.13 mol.dm^{-3} ammonium acetate adjusted to pH 9.0 with NH_3. Detection: electrochemical. Temperature: 30°C. Key: 1, tyrosine; 2, 3-hydroxykynurenine; 3, 5-hydroxytryptophan; 4, tryptophan.

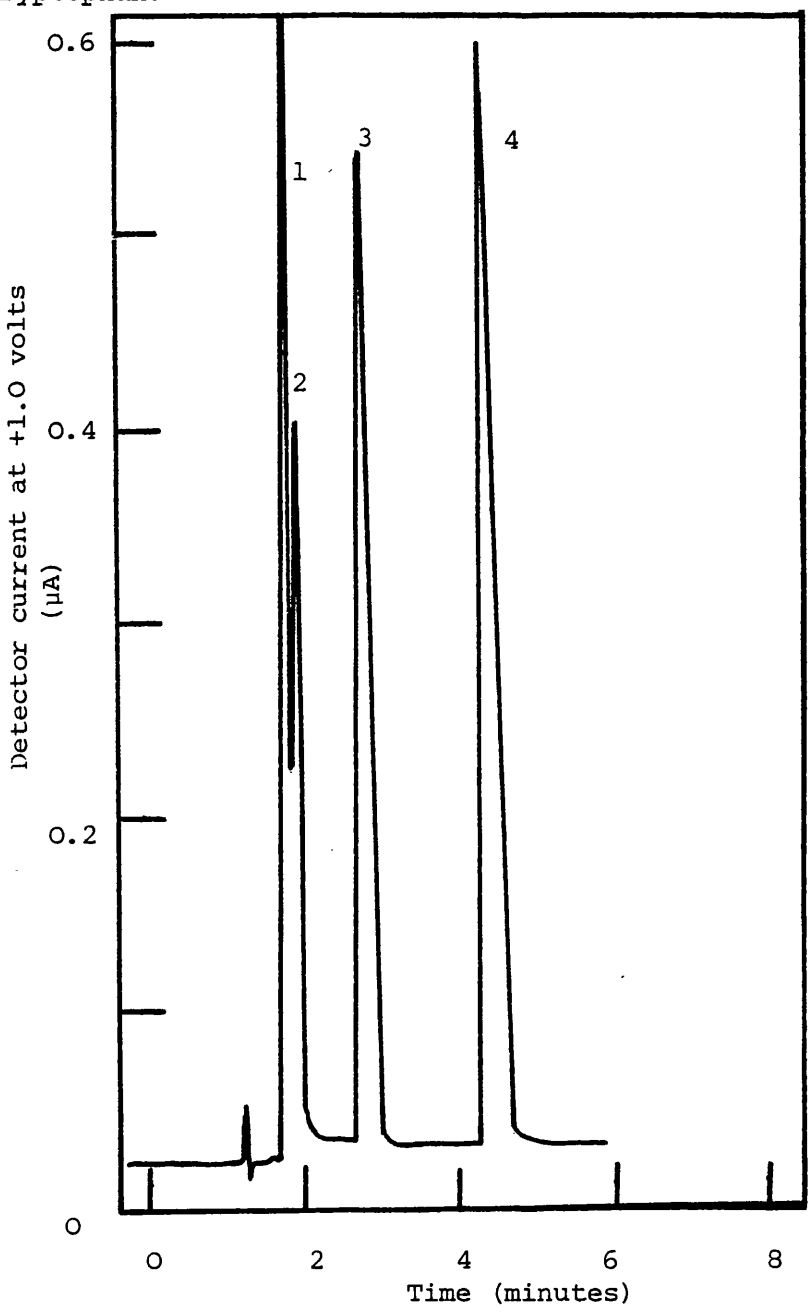


Fig. 4.72. Separation of four amino-acids showing the effect of D,L-carboethoxyheptyltrimethylammonium methane sulphonate (5×10^{-3} mol.dm^{-3}) (cf.Fig. 4.71). Key and other chromatographic conditions as Fig. 4.71.

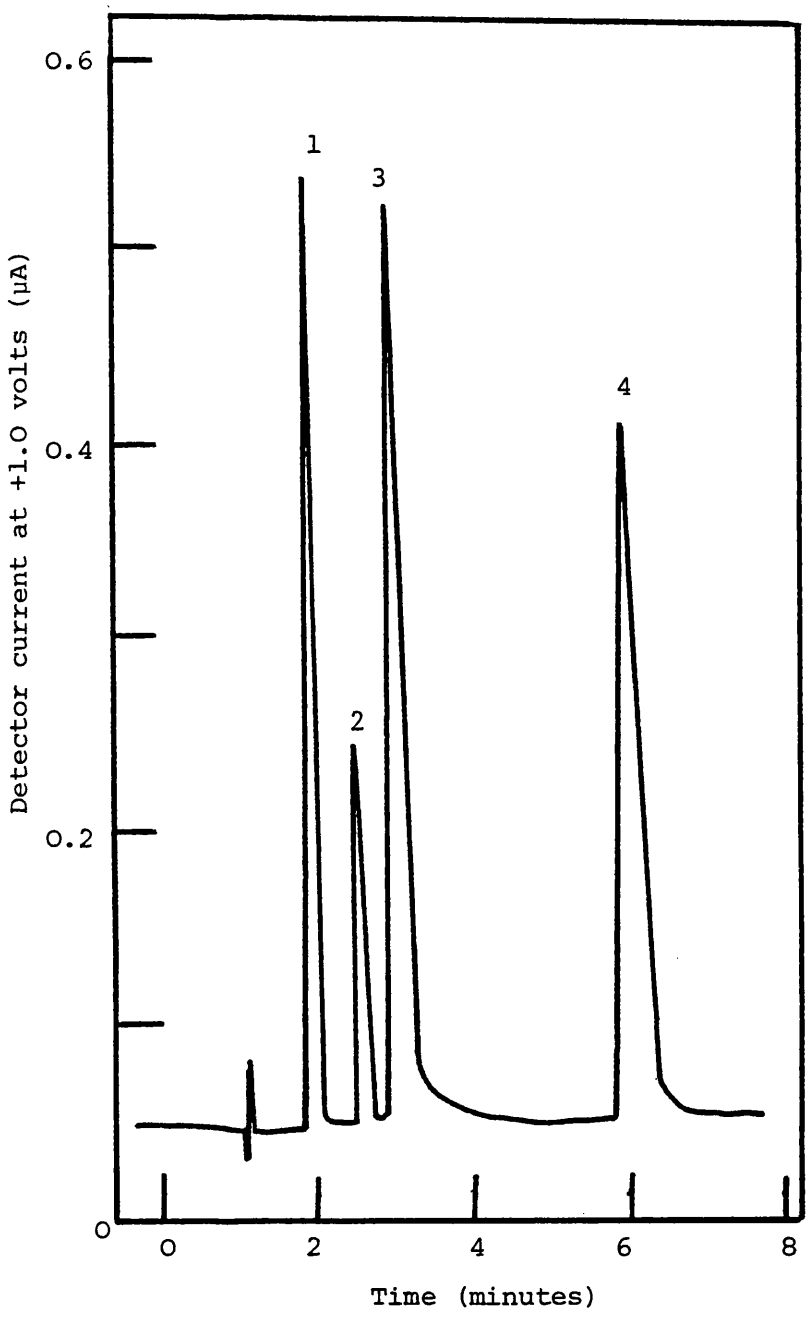
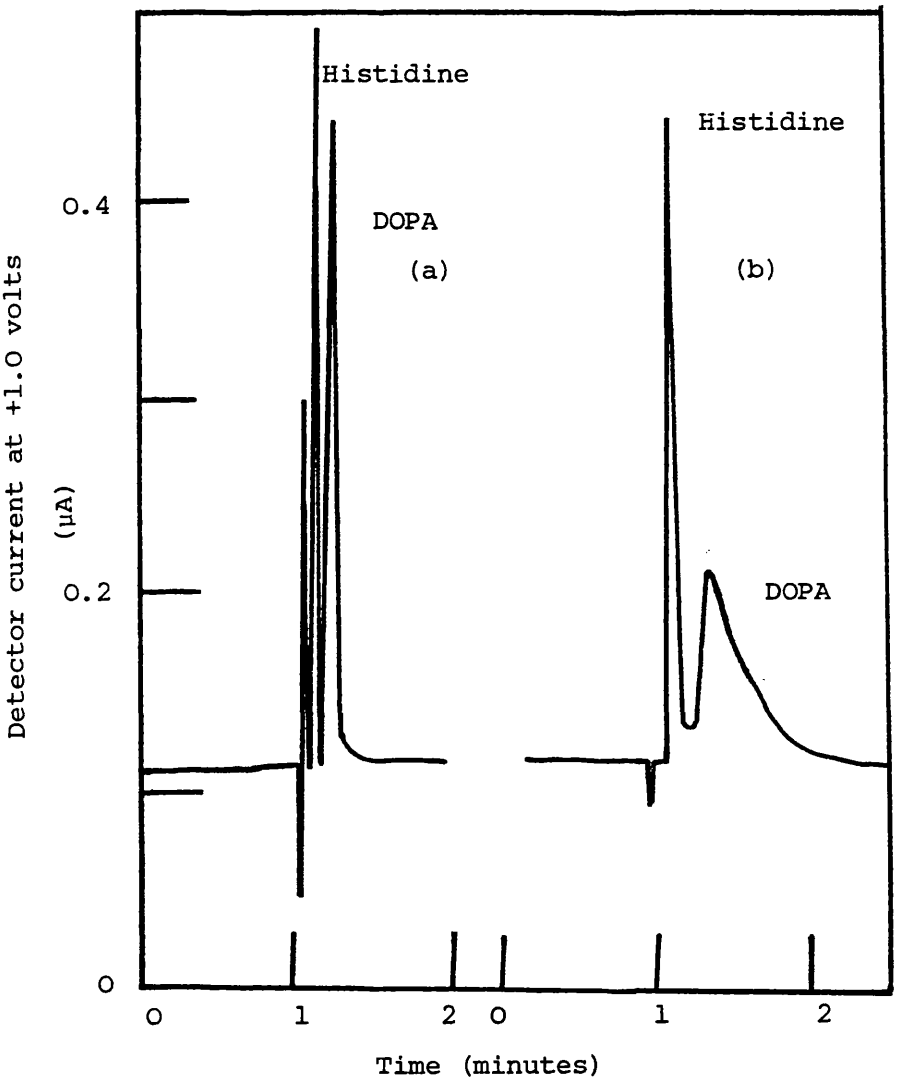


Fig. 4.73. Chromatograms of histidine and DOPA showing the effect of D,L-carboethoxyheptyltrimethylammonium methanesulphonate. Conditions: (a) as Fig. 4.71, (b) as Fig. 4.72.



SECTION 5. CONCLUSIONS

SECTION 5 CONCLUSIONS

The role of a pairing-ion in reversed phase HPLSC is firstly to reduce the net charge on the solute ion and secondly to increase the overall hydrophobicity of solute such that it is less solvated by the polar mobile phase and will hence be expelled to a greater extent from its aqueous environment. Increasing the concentration of the pairing-ion increases the extent of ion-pair formation; increasing the hydrophobicity of the pairing-ion increases the hydrophobicity of the formed ion-pair resulting in a net increase in retention. However this study shows that these two factors do not affect selectivity, indicating that the factors which control selectivity in ion-pair systems are the same as those which control selectivity in non ion-pair systems, (which was confirmed by the independence of τ on mobile phase pH). Solute selectivity was related to solute hydrophobicity since both τ and ion-pair distribution constants (ΔK_4) were correlated with bulk phase hydrophobicity parameters (e.g. π and Σf).

The dependence of solute retention and selectivity on hydrophobicity allowed many of the observations to be rationalised within the framework provided by Solvophobic Theory as suggested by Horváth *et al.* (87, 88). For example retention and functional group behaviour are found to be related to mobile phase surface tension, (although this dependence is perturbed by solute-solvent interactions, arising from van der Waal's interactions, in the cases of polar functional groups (e.g. OH, NH_2 and NO_2) as can be predicted from Solvophobic Theory. Solvophobic groups (e.g. Cl and CH_3) showed no dependence on organic modifier type but were solely dependent on mobile phase surface tension since Van der Waal's

interaction for such groups are minimal.

The dependence of the term B, K_2K_4 (Eq. 52) on solute hydrophobicity arises from the independence of the ion-pair formation constant (K_2) on solute structure and is only apparently dependent on solute charge invoking a stoichiometric effect. A notable exception to this is found in the cases of the 2-substituted solutes which have lowered ion-pair formation constants and resultant reduced τ values which are found to be unrelated to solute hydrophobicity. This indicates that steric and intramolecular effects perturb the general relationships and that ion-pair formation constants can influence retention and selectivity provided that either the solute or the pairing-ion are substituted close to their respective charge centres. The relevance of this steric effect on ion-pair formation is indicated in the separation of optical isomers by reversed phase systems involving ion-pairing effects. Additionally it is found that 2-substituted ion-pairs are solvated differently by different organic modifiers, which is shown to be a useful property for exploitation in the resolution of 2- and 4-substituted geometric isomers.

It is found that functional groups, (with the exception of the 2-substituents for the reasons described above), exhibited linear enthalpy-entropy compensation ,(as determined usingΔH-ΔG coordinates), suggesting a common retention mechanism for the 3- and 4-substituted benzoic acids. The plot obtained here for an ion-pair system was found to be coincidental with a similar plot obtained elsewhere (232) for neutral solutes confirming the hypothesis that the same solvophobic effects govern retention in

both ion-pair and non ion-pair systems.

Consistent with other studies for neutral solutes (76, 77) it is found that both retention and selectivity are dependent on stationary phase carbon loading and alkyl chain length, however this dependence is found to be confined to τ values for polar groups. This result tends to confirm the suggestion of Lochmuller and Wilder (76) that while Solvophobic Theory provides an adequate framework for the description of mobile effects in reversed phase HPLC it does not adequately describe the contribution of the stationary phase to selectivity.

The two contemporary theories of the retention mechanisms in reversed phase ion-pair systems using large hydrophobic pairing-ions are that the ion-pair forms either in the bulk mobile phase or at the hydrophobic surface due to the interaction of the solute with the adsorbed pairing-ions. Past studies (145, 129, 106, 107, 134) have concluded that when small organic or inorganic pairing-ions are used and there is no surface-excess of pairing-ion, that the solutes are retained by ion-pair formation and distribution. When the hydrophobicity of the pairing-ion is such that there is a surface excess it has been assumed (120, 80, 161, 163) that the mechanism changes from ion-pair distribution to one of *in situ* ion-exchange (i.e. ion-pair formation at the surface), which has been confirmed by an inability to detect ion-pairs in the mobile phase (161), or by anomalously high chromatographically derived ion-pair formation constants (80). *A priori* it is to be expected that an increase in the hydrophobicity of the pairing-ion will increase the extent of ion-pair formation, and this apparent contradiction arises from the assumption that

the reversed phase system behaves in an identical manner to bulk phase systems and does not take into account the significant contribution of the interface.

If ion-pairs do not form in the bulk mobile phase they will not form at the surface unless the environment of the adsorbed pairing-ion is noticeably different from the environment of the mobile phase, which in reality is the case since the charged head groups of the adsorbed surfactants do not protrude into the mobile phase but are situated within the interface close to the hydrophobic surface. Since it was shown that water structuring effects are diminished in methanol-water systems it is relevant to discuss ion-pair formation in terms of solvent dielectric. Since the surface of the stationary phase is coated with adsorbed organic modifier (75, 81, 80) at first sight it might appear reasonable to assume that the dielectric at the surface is close to that of pure methanol. Mukerjee and Ray (264) have shown that the dielectric at the surface of CTAB micelles in water is about 36 due to the high salt concentration in the interface and the structured nature of the solvent, and hence it is reasonable to assume that the dielectric at the surface of a hydrophobic stationary phase covered with surfactant and methanol is less than that of pure methanol.

Within the mobile phase/stationary phase interface there will be a concentration gradient of pairing-ion and organic modifier which will result in a corresponding dielectric gradient. This indicates that there are not merely two potential sites for ion-pair formation but several, and that the position at which ion-pairs

can form to any extent will depend upon their character, and that the chromatographically derived ion-pair formation constants will represent an average value. This hypothesis is confirmed here by the fact that derived ion-pair formation constants are relatively independent of solute structure and mobile phase environment. For example the K_2 values for 3 aminobenzoic acid based ion-pairs are 1488 $mol.^{-1}dm^3$ and 1983 $mol^{-1}dm^{-3}$ in mobile phases containing 50% methanol and 10% methanol, despite the latter having a higher solvent dielectric. The former value is consistent with significant ion-pair formation in the mobile phase environment whereas the latter is consistent with ion-pair formation in the interfaee but is too low to be consistent with ion-pair formation at the surface.

Very recently Bidlingmeyer *et al.* (265) reached a similar conclusion regarding the mechanism of retention of cationic solutes in reversed phase systems containing alkylsulphonate pairing-ions. These workers found evidence for neither a true ion-exchange process nor ion-pair formation in the mobile phase (methanol:water) in the system studied, and concluded that the retention is broader in scope (*sic*) than either of these two.

In their model, it is assumed that the pairing-ions are adsorbed onto the stationary support as a primary layer below a secondary layer containing the pairing-ions' counter ions (i.e. Na^+) and a dynamic equilibrium exists such that the pairing-ions are continually absorbing and desorbing through the secondary layer. Retention of an oppositely charged solute in this model of Bidlingmeyer *et al.* (265) is attributed to "ion-interaction"

(although not necessarily ion-pair formation) with the desorbing or absorbing pairing-ion in the secondary layer where ordinarily it would be electrostatically repelled.

The difference between the model proposed in this present study and that proposed by these workers is that in the former the interface is considered to be a heterogeneous continuum whereas in the latter the interface is considered to comprise of two homogeneous layers.

In addition to the application of Solvophobic Theory, it is found that retention and functional group behaviour could be rationised using both linear free-energy relationships and empirical log-linear plots of capacity ratio against organic modifier volume fraction. Slopes of these latter plots are found to be related to the extracting ability of the organic modifier and, at least in the case of methanol and propan-2-ol to solute hydrophobicity. The slopes of these plots for tetrahydrofuran and acetonitrile are found to be less dependent on solute hydrophobicity and are perturbed by specific solute-solvent effects (i.e. specific solvation). The functional group values can be well correlated with π values, with the slope coefficients for the relationship increasing with increasing water content of the mobile phase, and these correlations are perturbed by specific solvation effects when using non-alcoholic organic modifiers.

It has been shown that τ values may be used either to indicate hydrophobicity properties *per se*, or to be used in drug design models. Some advantages of HPLC over bulk-phase partition

studies in this respect are speed of determination, reliability, and also absolute purity of the solute is not necessary. The major disadvantage of such chromatographic determinations is the range of capacity ratios and hence solute hydrophobicity which may be determined in any one phase system. Consequently some scaling factor is required if measurements are to be made within a series of solutes of wide physicochemical properties. It has been suggested (184) that changing the organic modifier concentration and extrapolating the data to 100% water may be an appropriate scaling procedure. It has been shown here that this approach can lead to artifacts, particularly if non-alcoholic modifiers are used, Such artifacts arise from the non-linear behaviour of Log κ and τ with large organic modifier concentration ranges. A more appropriate scaling factor in the case of ionised solutes would be the concentration and hydrophobicity of the pairing-ion which may be varied independently of τ. In any one phase system the range of τ values which may be determined accurately is about 3 log units. By changing the concentration and type of the pairing-ion the capacity range of the reference compound is also 3 log units hence the accessible range of τ values using an ion-pair method is, in total, 6 log units. Additionally if the methanol content of the mobile phase is maintained at a fairly high value (e.g. 50%) the range of π values which may be predicted is about 12 log units since the slope coefficient of the τ versus π plot is about 0.5.

Linear free energy relationships may be used not only to rationalise retention mechanism but also to predict retention, but conversely to identify the structures of solutes when authentic samples are unavailable. This may be done by reference

either to published hydrophobicity parameters or more usefully, to predetermined τ values. In this study such a principle has been applied successfully in the separation of a series of different solutes such as drug degradation products, structurally related compounds and metabolites.

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APPENDICES

Suggestions for Future Work

This present study has shown that in general retention and selectivity in reversed phase ion-pair systems can be described by the application of Solvophobic Theory (89) although in common with non ion-pair systems this theory does not adequately describe the role of the stationary phase. Additionally this present study and that of Bidlingmeyer *et al.* (265), have shown that under certain conditions retention may be due to ion-pair formation in the stationary phase-mobile phase interface. Hence further studies are needed to investigate the roles of the stationary phase and the interface in reversed phase ion-pair systems and a potential approach might be to apply the methodology of Mukerjee and Ray (264) who investigated the interactions of ionised solutes with the surface of oppositely charged micelles.

The resolution of optical isomers by liquid chromatography employing ion-pair techniques is a clear objective and this present study has indicated that more work is required into the synthesis of sterero-specific optically active pairing-ions, and such separations may be enhanced further by the use of high efficiency columns such as the microbore columns described recently by Scott and Kucera (361, 362).

It has been demonstrated here that reversed phase ion-pair HPLC may be used to generate hydrophobicity parameters (τ) and that this technique has advantages over non ion-pair systems since changing the type and concentration of the pairing-ion allows a larger range of group hydrophobicity to be examined. This technique is restricted to ionic solutes and reliable scaling factors are required in non ion-pair

systems so that liquid-liquid distribution data from neutral solutes of wide physicochemical character may be obtained using reversed phase HPLC.

Chromatographic functional group values (τ) may also be used in the assignment of structures to peaks when authentic samples are unavailable which is often the case in drug metabolism studies, and the preliminary studies on the tryptophan metabolites need to be extended to produce a more complete data set of chromatographic enzyme values (τ_e)

This present study has demonstrated that HPLC is not only a powerful analytical tool but also has great potential in generating physicochemical data for drugs and organic compounds and the potential of HPLC in this latter respect needs to be assessed further in such areas as solubility, complexation (e.g. charge-transfer complexes) and drug-receptor binding. It is possible that the kinetics and affinities of drug-receptor interactions could be determined by binding the isolated protein receptor to an inert support and observing the retention behaviour of drug molecules, alternatively drug molecules could be bound to the stationary support and retention behaviour of the isolated protein observed.

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APPENDIX

Pairing-ion systems and solutes reported in the literature.

Pairing-ion	Nominal position of the pairing-ion	Solute	Reference
Alkylammonium ions			
Tetramethylammonium	M	Alkylbenzenesulphonates	266
	M	Hydrocortisone and salts	267
Tetraethylammonium	S	Phenylacetic acids	130
	S	Tricyclic antidepressants	268
	M	Hydrocortisone and its salts	267
	M	Tartrazine and its salts	132
Tetrapropylammonium	M	Hydrocortisone and its salts	267
	M	Steroidal glucuronides	269
	M	Carboxylic and naphthylacetic acids	270
Tetrabutylammonium	S	Carboxylic acids	271
	S	Sulphonamides	140, 141, 272
	S	Glucuronides and oestrogens	129
	M	Indoleacetic acids	273
	M,S	None	161
	M	Carboxylic acids	80
	M	Phenylacetic acids	130
	M	Ascorbic acid	159
	M	Tartrazine and its salts	132
	M	Hydrocortisone and its salts	267
	M	Cephalosporins	274
	M	Opiates and other drugs of abuse	275

Pairing-ion	Nominal position of the pairing-ion	Solute	Reference
	M	Folic acid derivatives	276
	M	Iodoamino acids	251
	M	N-substituted pteroylglutamic acids	277
	M	Sulphonamides and barbiturates	278
	M	Paracetamol metabolites	279
	M	Carbazoles	280
	M	Nitrophenols	281
	M	Benzoic acids	129
	M	Sulphonic acids and nicotinic acid	134
	M	Mandelic, nicotinic and acetic acids	282
	M	Anthraquinone glycosides	283
	M	Iodobenzoic acids	284
	M	3'-phosphoadenylsulphate and related compounds	285
	M	Acetyl coenzyme A thioesters	286
	M	Guaiacolsulphonate	287
	M	Bilirubin azopigments	288
	M	Pentachlorophenol	289
	M	EDTA	290
	M	Muscle relaxants and analgesics	291
	M	Amino acid conjugates	292
Tetrapentylammonium	S	Phenylacetic acids	130
	S	Glucoronides and oestrogens	129
	M	Hydrocortisone and salts	267
Inorganic and buffer ions			
Perchlorate	S	Antiarrhythmic	293
	S	Amitriptyline and nortriptyline	294
	S	Quinidine	295

Pairing-ion	Nominal position of the pairing-ion	Solute	Reference
	S	Thyroid hormones	141
	S	Catecholamines	271
	S	Phenothiazines and dibenzapenes	158
	S	Catecholamines	296
	S	Amino acids and biogenic amines	297
	M	Tetracyclines	298
	S	Adrenaline and noradrenaline	299
(+tri-n-octylamine(S))	M	Sulphonic and carboxylic acids	200
	M	Antiinflammatory drugs	300
	M	Carboxylic and sulphonic acids and phenols	301
Chloride	S	Amino acids	297
	S	Phenothiazines	302
	M	Emepronium and derivatives	303
Bromide	M	Emepronium and derivatives	303
	M	Organic ammonium ions	304,305
Phosphate	M	Iodoamino acids	251
	M	Peptides	306
	M	Hydrophobic amines	304,305
	M	Catecholamines and derivatives	156
	M	Acebutolol and metabolites	307
	M	Paraquat and diquat	154
	M	Amino acids, peptides and proteins	253

Pairing-ion	Nominal position of the pairing-ion	Solute	Reference
Nitrate	M	Erythromycin, diamines, benzocaine and amprolium	308
	M	Catecholamines and derivatives	156
Acetate	M	Tropane alkaloids	309
	M	Catecholamines and derivatives	156
Sulphate	M	Catecholamines and derivatives	156
	S	Aminopyrine and codeine	310,311
Trichloroacetate	M	Benzomorphans and methadone	312
	M	Catecholamines and derivatives	156
Formate	M	Erythromycins, diamines and amprolium	308
Picrate	S	Acetylcholine and choline	143,144
	S	Alkaloids	146,147,313, 314,315
	S	Alkylammonium ions	72
	S	Quaternary ammonium ions	128,136
Alkylsulphonates			
Methylsulphonate	M	Tricyclic antidepressants	316,317
	M	Catecholic amino acids	318
	M	Tris (2,2'-bipyridyl)-ruthenium (II)	319
Butylsulphonate	M	Pyrilamine	320

Pairing-ion	Nominal position of the pairing-ion	Solute	Reference
Pentylsulphonate	M	Diminazene derivatives	321
	M	Elipticene	322
	M	Methadone	323
Hexylsulphonate	M	Adrenaline	324
	M	Peptides	249
Heptylsulphonate	M	Niacin and niacinamide	159
	M	Opium alkaloids	325
	M	Paracetamol metabolites	326
	M	Elipticene	322
	M	Atenolol and metoprolol	327
	M	Bleomycins	328
	M	Diazonium salts	329
	M	Anticholinergics	145,330,331
	M	Thyrotrophin releasing hormones	332
	M	Dansylated urinary polyamines	333
	M	Catecholamines	334
	M	Antihistamines	335
	M	Opiates	275
	M	Xanthines	336
	M	Mercaptopurine	337
	M	Catecholamines	338
	M	Catecholamines and antihistamines	339
	M	Benactyzine	340
	M	N,N'-trimethylene-bis(pyridinium-4-aldoxime) dibromide	341
Octylsulphonates	M	3-pyridylcarbinol and 6-amino-3-cresol	342
Toluenesulphonate	M	Quinidine	343

Pairing-ions	Nominal position of the pairing-ion	Solute	Reference
Camphor-10-sulphonate	M	Niacin and niacinamide	344
	M	Peptides	255
	M	Amino acids	This study
Dioctylsulphosuccinate	M	Isoniazid and pyridoxime	345
	M	Niacin and niacinamide	159
Cyclohexylsulphamates + long chain alkylammoniums	M	Organic ammoniums	304,305
Indicating ions (see also picrate)			
Naphthalene-2-sulphonate	S	Amines	150
	S	Amino acids, peptides and alkylamines	151
N,N-dimethylprotriptylene	S	Aromatic acids	148,136
	S	Alkylsulphates and sulphonates	152
N-methylimipramine	S	Aromatic acids	149
9,10-dimethoxyanthracene-2-sulphonate	S	Basic drugs	346
	M*	Basic drugs	347

* post column segmented flow

Pairing-ion	Nominal position of the pairing-ion	Solute	Reference
Surfactants			
Octylsulphates	M,S	Organic ammonium ions	296
	M,S	Atenolol and related compounds	This study
	M,S	Propanolol	This study
	M,S	Amines	162
	M,S	Catecholamines	348
Decylsulphate	M,S	Amines	162
Dodecylsulphate	M,S	Pyridostigmine	349
	M,S	Catecholamines and derivatives	350
	M,S	Pentapeptides	164
	M,S	Amino acids	165
	M,S	Tryptophan	This study, 351,247
	M,S	Synthetic peptides	This study
	M,S	Butyrophenones and diphenylbutyl-piperidine	166
	M,S	Catecholamines	266,296
	M,S	Amino acids, peptides and proteins	253
	M,S	Blufomedil hydrochloride	352
	M,S	Hydroxyphenylglycine isomers	This study, 234
Octylsulphonate	M,S	Pyridoxime	161
	M,S	Catecholamines and amino acids	353
	M,S	Catecholamines	266,296
	M,S	Phenylephrine and lidocaine	354
Dinonoylnaphthalene-sulphonate	M,S	Amino acids	165

Pairing-ion	Nominal position of pairing-ion	Solute	Reference
Dodecylbenzenesulphonate	M,S	Catecholamines	296
ABDACs	M,S	Benzoic acids	This study, 218, 355, 356
	M,S	Acid dyes	357
	M,S	6-chlorocinnoline-3-carboxylic acid	This study
	M,S	Sodium cromoglycate	This study, 213,218
Tridecylammonium	M,S	Hydrocortisone and salts	267
Hexadecyltrimethylammonium (CTAB, Cetrimide)	M,S	Labetolol	358
	M,S	Sulphonic acids and dyes	120
	M,S	Sulphonic acids	72
	M,S	Carboxylic and sulphonic acids	167
	M,S	Penicillins and cephalosporins	359
	M,S	Inorganic anions	360
Dioctylammonium	M,S	Paracetamol metabolites	279